THE IMPACT OF ENZYMATIC HYDROLYSIS ON THE NUTRITIONAL AND FUNCTIONAL PROPERTIES OF AN AIR-CLASSIFIED PEA PROTEIN-ENRICHED FLOUR

A Thesis Submitted to the College of
Graduate and Postdoctoral Studies
in Partial Fulfillment of the Requirements
of the Degree of Master of Science
in the Department of Food and Bioproduct Sciences
University of Saskatchewan
Saskatoon, Saskatchewan, Canada

By
Dellaney Konieczny
2019

PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a Postgraduate degree from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis work or, in their absence, by the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying, publication, or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Requests for permission to copy or to make other use of material in this thesis in whole or part should be addressed to:

Head Department of Food and Bioproduct Sciences
University of Saskatchewan
Room 3E08 Agriculture Building, 51 Campus Drive
Saskatoon, Saskatchewan S7N 5A8
Canada

OR

Dean
College of Graduate and Postdoctoral Studies
University of Saskatchewan
116 Thorvaldson Building, 110 Science Place
Saskatoon, Saskatchewan, S7N 5C9
Canada

ABSTRACT

The overall goal of this research was to improve the protein quality and functionality of air-classified protein-enriched flour (pea protein-enriched flour; PPEF) using different enzymes and levels of hydrolysis. Initially, PPEF was hydrolyzed using seven different enzymes, and based on the initial degree of hydrolysis (DH) results, four were chosen for further investigation (Savinase, trypsin, pepsin and papain). The conditions of hydrolysis (time, temperature, pH and enzyme substrate ratio) were altered to modify the protein to have degree of hydrolysis of 2-4% and 10-12%. Each of the chosen enzymes has different substrate specificities and preferred solvent conditions, which were hypothesized to lead to differences in protein unfolding, surface characteristics, functionality, and protein quality.

The physicochemical (surface hydrophobicity and charge) and functional properties (solubility, oil and water holding capacity, emulsification and foaming) were initially examined for hydrolyzed PPEF as a function of enzyme type and DH. The surface hydrophobicity was found to increase from 13.3 A.U. (arbitrary units) in the untreated flour to between 22.8 and 48.5 A.U. in the hydrolyzed flours, with the greatest increase occurring with papain hydrolysis. The surface charge of untreated PPEF was found to be -12.6 mV, whereas it became more negative with hydrolysis (ranging between -14.0 to -19.0 mV) with the greatest increase occurring with the pepsin treatment. The emulsion activity and stability index of the untreated flour was higher at all pH values tested compared to the hydrolyzed flours, regardless of the enzyme used. A similar trend occurred for foaming capacity and stability, as well as solubility parameters. In contrast, water (WHC) and oil (OHC) holding capacities were found to both increase with hydrolysis. For instance, WHC increased from 0.6 g/g to 1.4-2.0 g/g following hydrolysis, with the greatest improvement occurring using the papain treatment; whereas, OHC increased from 0.7 g/g to 1.0-1.5 g/g following hydrolysis, with the greatest improvement occurring using papain.

Changes to the levels of bioactive compounds (total phenolics, condensed tannins, trypsin and chymotrypsin inhibitors) within the PPEF with hydrolysis was also investigated. The total phenolic contents (gallic acid equivalents; GAE) were found to be reduced from 8.1 to 5.4-7.1 mg GAE/g following with pepsin being most effective hydrolysis treatment. Similarly, condensed tannins were reduced from 0.7 mg catechin equivalents/100g to values that were undetectable by the assay for all enzymes and DH. In addition, both protease inhibitors decreased in concentration

(*i.e.*, chymotrypsin inhibitor units (CIU) and trypsin inhibitor units) with hydrolysis. For instance, chymotrypsin inhibitors were reduced from 63.9 to 3.5-7.1 CIU/mg following hydrolysis, with the greatest decrease observed with the papain treatment. Trypsin inhibitors were reduced from 38.4 to 9.9-17.3 TIU/mg, with the greatest decrease observed with Savinase. The protein quality of untreated and hydrolyzed PPEF was also analyzed by determining the amino acid score, *in vitro* protein digestibility (IVPD) and *in vitro* protein digestibility corrected amino acid score (IVPDCAAS). Methionine and cysteine remained the limiting amino acids in the PPEF for all hydrolysis treatments. However, the limiting amino acid score was found to improve from 0.80 for the untreated flour to 0.79-0.84 with 10-12% DH, and have a lower AAS with lower levels of hydrolysis (DH 2-4%), 0.66-0.72 for trypsin and papain, with pepsin having the greatest improvement to 0.84. The IVPD increased from 83.9% in the untreated flour to 85.5-88.8% following hydrolysis, with the greatest improvement occurring with the papain treatment. IVPDCAAS of the untreated flour was determined to be 67.7%, which then declined with 2-4% DH (59.2 to 64.6) before increasing at higher levels of hydrolysis (DH 10-12%) (68.6 to 72.9), with the greatest increase occurring with pepsin treatment. It was observed that hydrolysis was found to improve the nutritional quality of the PPEF based on the lower amounts of bioactive compounds and higher IVPDCAAS values.

Overall, enzymatic hydrolysis improved non-solubility dependent functional properties (WHC and OHC), with 10-12% papain-hydrolyzed samples having the most improvement. Improvements in WHC and OHC could mean the ingredient could be applied into baked goods or used as a meat binder. However, increases in surface hydrophobicity and low zeta potential may have led to decreases in solubility-dependent functional properties (EAI, ESI, FC, FC) as solubility decreased with all hydrolyzed samples. The bioactive compounds decreased with all enzymatic treatments, and the IVPDCAAS was increased with 10-12% DH with the greatest improvement using pepsin treatment. Enzymatic hydrolysis as a means of protein modification could improve the functional and nutritional properties for a value-added PPEF ingredient.

ACKNOWLEDGEMENTS

I would like to offer my sincere gratitude to both of my supervisors Dr. Michael Nickerson and Dr. Takuji Tanaka for offering me the opportunity to further my studies at the University of Saskatchewan. Their academic guidance and encouragement was greatly appreciated throughout my program. I would like to thank my advisory committee, Dr. Darren Korber, as well as my past and present committee chairs, Drs. Supratim Ghosh and Chris Eskiw, for their valuable advice throughout my project. I also wish to thank my external examiner, Dr. Brian Bandy, for the time taken to review and provide feedback on this thesis.

I would like to express my sincere thanks to Dr. Burcu Cabuk, Andrea Stone, and Dr. Nicole Avramenko for offering training and technical assistance throughout my research, and academic support during my research. I would also like to thank Dr. Matthew Nosworthy and James House from the University of Manitoba for helping with the protein digestibility analysis and providing nutrition and protein quality insights. I would like to express my appreciation to Donna Selby and Ann Harley for administrative support throughout the process as well as the faculty, staff and students in the Department of Food and Bioproduct Sciences. I especially would like to thank Hayley Kumitch for the friendship and assistance during our graduate studies.

I wanted to extend a special thank you to my friends and family and especially my mom Mary Konieczny who was always there to listen throughout both the good and difficult times with reassuring words. Finally, I would like to thank Travis Klym for all the support and encouragement and Ellis and Donna Treffry for their inspiration, and kind words.

Financial support for this study was provided by the Global Institute for Food Security and the Saskatchewan Agriculture Development Fund.

TABLE OF CONTENTS

LIST OF TABLES

Page Number

LIST OF FIGURES

Page Number

LIST OF EQUATIONS

Page Number

LIST OF SYMBOLS AND ABBREVIATIONS

AA	Amino Acids
AACC	American Association of Cereal Chemists
AAS	Amino acid score
ANOVA	Analysis of variance
ANS	8-Anilino-1-naphthalenesulfonic acid
AOAC	Association of Official Analytical Chemists
CIA	Chymotrypsin inhibitor activity
CIU	Chymotrypsin inhibitor units
d.b.	Dry basis
DF	Dilution factor
DH	Degree of hydrolysis
DL-BAPNA	Na-benzoyl-D, L-arginine 4-nitroanilide hydrochloride
DMSO	Dimethyl sulfoxide
EAI	Emulsion activity index
EAA	Essential amino acid
[E/S]	Enzyme substrate ratio
ESI	Emulsion stability index
ε	permittivity
η	Dispersion viscosity
FAO	Food and Agriculture Organization of the United Nations
FC	Foaming capacity
FS	Foaming stability
$f(\kappa\alpha)$	Smoluchowski approximation
g	Gravitational Force
GAE	Gallic acid equivalents
h	hour
h	Yield of hydrolysis equivalents (of α-amino groups formed during hydrolysis reaction; or α-NH_2-Gly equivalent)

h_c	mM concentration of α-NH_2-Gly equivalent from untreated pea protein-enriched flour
h_t	mM concentration of α-NH_2-Gly equivalent from enzyme hydrolyzed pea protein-enriched flour hydrolysis
h_{tot}	mM concentration of α-NH_2-Gly equivalent from total pea protein-enriched flour hydrolysis
HPLC	High performance liquid chromatography
IVPD	*In vitro* protein digestibility
IVPDCAAS	*In vitro* protein digestibility corrected amino acid score
κ	Debye length
kDa	Kilodalton
MWM	Molecular weight marker
mAU	milliAnson unit
OHC	Oil holding capacity
Pa	Papain
Pe	Pepsin
PER	Protein efficiency ratio
pI	Isoelectric point
PPEF	Pea protein-enriched flour
PDCAAS	Protein digestibility corrected amino acid score
pH	Acidity in logarithmic scare
S	Savinase
S_0-ANS	Surface hydrophobicity
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
T	Trypsin
TIA	Trypsin inhibitor activity
TIU	Trypsin inhibitor units
TCA	Trichloroacetic acid
TNBS	2,4,6-trinitrobenzenesulfonic acid
U_E	Electrophoretic mobility

WHC	Water holding capacity
ZP	Zeta potential
ζ	Zeta potential

1. PROJECT OVERVIEW

1.1 Introduction

The Food and Agriculture Organization of the United Nations (FAO) estimates that the global population will increase to ~9 billion people by the year 2050. As such, countries will need to increase their food production by 70% to meet the growing demand (FAO, 2013). In terms of ensuring a secure food supply of high-quality protein, researchers and policy makers are looking towards sustainable food production practices with reduced environmental impacts (Sabate & Soret, 2014). Increasing the production of peas is one (of many) promising strategies for achieving a stable food source of high-quality protein, since it produces more protein per land mass than animals, requires less fertilizer and water for growth than other plant sources, and "fixes" atmospheric nitrogen within the soil (Vankosky *et al.*, 2011; Sabate & Soret, 2014). Protein ingredients are derived from a range of sources from animals, plants, microalgae, insects and fungi. Animal-derived proteins from milk (casein and whey), eggs (ovalbumin) and bovine/porcine (muscle/connective tissue proteins) are dominant protein sources currently on the market. However, plant-based proteins (other than wheat and soy which have allergen concerns) are gaining popularity (Day, 2013).

Field pea is an important grain legume that is already widely-grown and consumed around the world. Peas, like other pulses are high in proteins and carbohydrates, as well as vitamins and minerals needed in the human diet (Foegeding & Davis, 2011). Their proteins tend to be high in lysine, but deficient in the thiol-containing amino acids (cysteine and methionine). As such, they are often consumed alongside of cereals to provide a complementary source of essential amino acids (Pulse Canada, 2016). Pulses are the edible seed portions of the legume pod, which are dried, cooked and then consumed. In Canada (mostly Saskatchewan, Alberta and Manitoba) there is an abundant supply of peas as the climate and soil conditions are well suited for their growth. Peas grown in Canada are exported (6 million tons in 2015) all over the world and have a significant role as an ingredient for the canning industry, bakery products, protein meal replacements, pasta and vegetarian products (Day, 2013; Pulse Canada, 2016). Pea proteins are considered non-

genetically modified, abundant, low in cost and have lower allergenicity than wheat or soy (Fredrikson *et al.*, 2001; Boye *et al.*, 2010). They also have excellent functional properties (*e.g.*, solubility emulsification, foaming, oil/water holding and gelation), but often require some levels of minimum processing to improve their properties and to reduce levels of bioactive compounds (*e.g.*, enzyme inhibitors, lectins, phytates, and phenolic compounds) which inhibit their digestion.

1.2 Hypotheses

In order to address the overall goal and objectives of this research, the following hypotheses were be tested:

a) enzymatic hydrolysis of pea protein-enriched flour can be optimized for partial protein modification through the use of specific or non-specific commercial enzymes by determining the optimum pH, [E/S] ratio and temperature on an air-classified pea protein-enriched flour (pea protein-enriched flour; PPEF); and

b) the functional properties (*i.e.*, solubility, emulsions, foaming and oil and water holding capacity) of the resulting enzymatically-treated PPEF will increase, due to the reduction in molecular weight, the change in conformation, the increase of hydrophobic and hydrophilic groups on the protein in addition to increasing the nutritional properties through enzymatic hydrolysis.

1.3 Objectives

The overarching goal of this research is to investigate the effect of partial enzymatic hydrolysis on the protein quality in a PPEF. In this case, protein quality refers to its functional properties, its amino acid composition and the bioavailability of essential amino acids during digestion. Specific objectives include:

a) optimizing the hydrolysis conditions (*i.e.*, enzyme choice, [E/S] ratio, pH, temperature, degree of hydrolysis) of PPEF; and

investigating the impact of partial enzymatic hydrolysis on:

b) the physicochemical and functional properties of PPEF;

c) the levels of bioactive compounds present; and

d) their *in vitro* digestibility.

2 LITERATURE REVIEW

2.1 Pea protein

The protein content in peas (*Pisum sativum* L.) ranges between 22-23% depending upon the environmental factors, growing conditions as well as the cultivar (Rubio *et al.*, 2014). Pea proteins are typically classified based on their solubility in different solvents, where globulins, albumins and prolamins are salt-, water- and alcohol-soluble, respectively (Shewry *et al.*, 1995). However, most proteins in pea consist of globulins and albumins. The former represents the major fraction (~65-80% of the total protein) and contains low levels of sulfur containing amino acids (*i.e.*, cysteine and methionine) (Day, 2013). The globulin proteins can further be sub-divided into legumin (11*S, S* is a Svedberg Unit), vicilin (7*S*) and convicilin (7*S*) proteins. Legumin is an 11*S* hexameric protein with molecular mass (MM) of 300-400 kDa, and is comprised of two monomers: an acidic (MM-40 kDa) and basic (MM-20 kDa) chain connected by a disulfide bond. Hexameric proteins are stabilized by hydrophobic forces, hydrogen bonds, and Van der Waals forces. In contrast, vicilin is a trimeric protein with a MM of 175-180 kDa (Rubio *et al.*, 2014). Each monomer of vicilin has a MM of 50-60 kDa and forms a trimeric structure. The composition of vicilin lacks cysteine residues, therefore disulfide bonds are unable to form; hydrophobic interactions, Van der Waals and hydrogen bonds are the main stabilizing forces (Day, 2013). Convicilin (MM ~290 kDa, subunits of ~71 kDa) is non-glycosylated and has sulphur amino acids which do not appear in the vicilin structure (Croy *et al.*, 1980). Albumin proteins consist of enzymes, protease inhibitors, lipoxygenase and lectins and range in MM between 14 and 80 kDa (Day, 2013).

2.2 Protein extraction

Pea seeds can be processed into three different protein products: flour, a protein concentrate and a protein isolate, using varied methods of extraction (*i.e.*, pin milling, air classification and wet extraction). These protein products then can further be processed into protein-rich foods such as meal replacements, bakery products and health supplements. The

various methods of protein extraction each have their advantages and disadvantages, and varies in both the quality and protein contents within the product. There are many variations in the protein contents yielded that can be attributed to various processing conditions (*i.e.*, temperature, pH, ionic concentration, flour: water ratio, pilot scale vs. commercial scale, etc.), therefore it is important to consider the method used based on the final product. The functional properties of protein are thus affected as well, and can be manipulated through different mechanisms of extraction influencing the food texture, and compatibility in the use of food products (Boye *et al.*, 2010).

Dry processes

Flours can be obtained from dehulled pea seeds using pin milling which grinds the seeds into a fine powder. Air classification can further separate flour into both starch-rich (heavier coarse fraction) and protein-rich (lighter fine fraction) fractions based on their size and density within a spiral air stream within the air classifier (Pelgrom *et al.*, 2013). The separation of starch from protein is enhanced because of the physical properties. Starch is heavy and dense while proteins are lighter and more jagged, which allows their separation from starch by air flow. The light particles (protein) are able to exit through an outlet, while the starch remains in the chamber due to its greater density (Reichert, 1982). The particle size, shape and density and aerodynamics of the pulse used are important factors in determining the separation of protein and starch. This process can be repeated as some starch remains adhered on the protein fraction. Gueguen (1983) noted that more than two-runs of air-classification does little to increase the protein content. The obtained protein from air-classification is considered an enriched flour (<65% protein on a dry weight basis) or protein concentrate (>65% protein on a dry weight basis), depending on the purity. Dry processing protein has many advantages, as the protein can retain its native functionality, and requires low energy and less water usage (Boye *et al.*, 2010; Pelgrom *et al.*, 2013). However, the purity of the protein concentrate obtained is low (~50 % for pea) (Boye *et al.*, 2010). Pelgrom *et al.* (2013) investigated the dry processing of milled peas (impact and jet) under various conditions to remove the starch from the protein. After air classification, concentrates have protein levels between 51-55%. The authors found that impact and jet milling at speeds of 4,000 rpm was best for separating starch from protein to give maximum protein recovery (76.8%). The authors suggested that milling at slower speeds couldn't extricate the starch from the protein, while extensive milling can damage the starch and lead to flour flow ability complications. As air-

classified pulse proteins are lower in starch and richer in protein than the flours, and as such make good candidates for the starting materials for wet processing to make concentrates or isolates. The use of pea protein concentrates has been studied to determine their effectiveness as an egg replacements in bakery products due to the high water holding capacity and their ability to form gels (Pelgrom *et al.*, 2013). Le Gall *et al.* (2005) investigated the effect of grinding, in addition to thermal treatment on pea proteins sensitivity to be hydrolyzed with pepsin and trypsin. The authors found that the bigger the particle size obtained from grinding, the less hydrolysis occurred. This finding suggests that the smaller the protein particle size obtained, the greater the ability of the protein to be hydrolyzed.

Wet processes

Wet processing is needed to obtain significantly-higher protein levels required to form a concentrate/isolate. Dry processes can only produce protein concentrates with 40-75% protein as discussed, whereas wet processes can produce isolates with 70-90% protein (Kiosseoglou *et al.*, 2011; Singhal *et al.*, 2016). However, wet processing can lead to a loss of native functionality due to fluctuating pH and drying (leading to potential denaturation). Wet processes can either be alkali- or acid-mediated, and followed by isoelectric precipitation or ultrafiltration. In addition, wet processes require significant amounts of water, as well as chemicals, which are disadvantageous as the industry strives for clean labels and processes. Wet processes depend on solubility of protein; therefore, insoluble proteins are excluded from the isolate, leading to significant losses. Wet processes can further be divided into isoelectric precipitation, salt extraction, micellularization and ultrafiltration.

Alkaline extraction - isoelectric precipitation: The most common method of obtaining pea protein concentrates/isolates is alkaline extraction followed by isoelectric precipitation (Fuhrmeister & Meuser, 2003; Boye *et al.*, 2010; Stone *et al.*, 2015). This method employs solubility manipulation of pea flour in water, where proteins are extracted under alkaline conditions (pH 8-9), clarified by centrifugation and then precipitated by adjusting the pH to near the protein's isoelectric point (pH 4.5-5). Once the protein is precipitated, it is centrifuged, neutralized and then spray-dried. Can Karaca *et al.* (2011) investigated the effect of emulsion formation of isoelectric precipitated and salt extracted protein isolated from various legumes. The researchers found that the legume protein (chickpea, faba, lentil and pea) resulting from isoelectric

precipitation had higher solubility and surface charge, thus increasing the emulsion forming capacity than that of salt extracted legume protein.

Salt extraction: Salt extraction is a process utilizing the "salting-in" and "salting-out" phenomena in proteins. Solubility of proteins can be enhanced by the addition of salt at low ionic strength with salts that promote structuring of the hydration layer or protein-water interactions over water-ion interactions (*e.g.*, sodium chloride) (known as salting-in) (Maurer *et al.*, 2011). Depending on the ionic strength, different types of protein are more soluble than others. Then in contrast to the solubilizing salt-in effect, proteins can be selectively-precipitated based on their hydrophobicity by introducing salts that disrupt the hydration layer surrounding the proteins by favoring water-ion interactions over protein-water interactions (*e.g.*, ammonium sulphate) (known as salting-out). Once the protein has been precipitated, it is centrifuged, neutralized and then spray dried. Sun & Arntfield (2010) researched how extraction of pea protein using a salt-extraction method influenced the gelation properties. The authors found that the salt-extraction process increased the gelation ability compared to that of isoelectric precipitation. The authors suggested that due to the mild-denaturation of pea protein resulting from the salt-extraction process, gelation abilities were increased.

Micellularization: Micellularization involves dissolving proteins within dilute NaCl solutions to solublize the proteins, and then adding 5-10 volumes of cold water to induce the formation of micelles. After a 24-h period under static conditions, a protein precipitate layer will form near at the bottom of the reaction vessel, which can be collected via centrifugation or ultrafiltration. Once the protein is precipitated, it is centrifuged, neutralized and then spray-dried.

Ultrafiltration: Ultrafiltration (UF) is a membrane-mediated method of extracting protein in an alkaline/acidic environment that yields an isolate with improved functionality, and potentially can remove some bioactive compounds (Klupsaite & Juodeikiene, 2015). The supernatant obtained from acid/alkaline extraction is passed through a membrane, yielding a more concentrated protein solution. The membrane used to separate proteins is selected based on the proteins size, where the membrane molecular weight limits range from 1 to 1000 kDa. Ultrafiltration relies on the pressure to drive the proteins through the filter, where the flow rate is determined by the membrane capacity and concentration of the protein solution. This can lead to problems in filtration as the membrane can become plugged and lead to a low flow rate. There are advantages to ultrafiltration such as milder operating conditions, leading to less losses and a higher yield than isoelectric precipitation.

Isolates obtained from ultrafiltration have a higher protein content compared to that of IEP (Boye *et al.*, 2010), and some functional aspects can be improved. UF has also been studied for producing pea proteins isolate with lower contents of bioactive compounds, such as oligosaccharides (Fredrikson *et al.*, 2001). They found that by using ultrafiltration to produce pea protein isolates for soy protein replacement in infant foods could be produced with superior nutritional properties. The authors suggested that UF can reduce the phytate and oligosaccharides, which improved the nutrient bioavailability, and reduces the flatulence suggesting that an ideal replacement for soy protein isolate can be made from pea protein.

Comparisons of extraction methods: There are many methods to extract pulse proteins that vary in the conditions used to process isolates. This can yield many different qualities, functional properties and compositions; therefore, determining the optimal extraction process is crucial to the final product obtained. Fuhrmeister & Meuser (2003) investigated two extraction methods (IEP and UF) on the functional properties of pea protein concentrate. The researchers found different functional properties based on the extraction process, and found superior functional properties (*i.e.*, emulsification, foaming, fat binding capacity) were obtained by UF compared to IEP. In addition, the researchers found UF led to a higher crude protein content than those produced by IEP. The authors attributed their results to UF producing a superior solubility pea protein concentrate as functional properties greatly depend on the solubility of the protein. Similarly, Boye *et al.* (2010) investigated how UF and IEP extraction methods influenced the functional properties of three legumes (pea, chickpea, and lentil) protein concentrates. The researchers found that both methods concentrated the protein contents 4-fold, however UF produced a concentrate with a higher protein content for all legumes tested. The functional properties exhibited by the legume concentrates were good, however certain treatments varied the extent of the functional properties. Ultrafiltration showed an increase in foam expansion, gelation, and solubility, while IEP increased water-holding capacity. Stone *et al.* (2015) investigated the impacts that salt-extraction, IEP, and micellar precipitation on the functional properties of pea protein. The authors found that salt-extracted protein had the highest protein solubility, oil holding capacity, foaming capacity, and emulsion capacity, while isoelectric precipitation had the highest hydrophobicity, foam stability. The emulsion stability was similar throughout all processes used with high emulsion stability. The investigators suggested that salt extraction had the best isolates in the yield obtained and the functionality produced.

2.3 Types of protein modification

The functional characteristics mentioned below (*i.e.*, solubility, water and oil holding capacity, emulsification, foaming, gelation) may improve or be inhibited through protein modification. These processes modify the protein's structure leading to conformational changes in the protein. This occurs by cleavage of the protein structure, yielding smaller peptides and free amino acids. In addition, the nutritional aspects of protein (*i.e.*, digestibility, increase in bioavailability) can be improved through modification. Methods of modification (*i.e.*, enzymatic, chemical and microbial) range in their severity, costs, and yield of hydrolysate and each has their advantages and disadvantages.

Chemical hydrolysis (*i.e.*, acid hydrolysis, alkaline hydrolysis) is a common method of hydrolysis of proteins in the food industry; however, it is one of the harshest processes and requires a significant amount of time. The process takes place at high temperatures, and extreme acidic, or alkaline pH (Tavano, 2013). Chemical hydrolysis leads to a high degree of hydrolysis (DH) impacting the functional and nutritional properties significantly. This high DH can be attributed to the low specificity of hydrolysis of protein. Acid hydrolysis occurs by cleaving peptide bonds at random, therefore the hydrolysis reaction is difficult to control. Chemical hydrolysis can also change the contents of amino acids. Acid hydrolysis can lead to high losses of certain amino acids especially tryptophan (Kasera *et al.*, 2015); whereas alkaline hydrolysis leads to a decrease in cysteine, arginine, threonine, serine, isoleucine and lysine (Tavano, 2013). In addition, acid hydrolysis can lead to undesirable side reactions that develop off flavours, texture, and potentially can impact the safety of the food and development of products (Kasera *et al.*, 2015).

Microbial modifications through fermentation of the protein is a method employing the use of bacteria or fungi that secrete proteolytic enzymes, which then hydrolyze peptide bonds in the protein (Sun, 2011). As fermentation progresses, by-products are produced and can in turn lead to off flavours or colours. Fungi can produce a wider array of enzymes than bacteria and can be used for many different applications. This method is one of the most economical and simple methods of hydrolyzing protein. However, the applications of protein fermentation have produced varying results (*i.e.*, enhanced or was detrimental) to functional and sensory properties.

Enzymatic modification uses the enzymes obtained from animal, vegetable or microorganisms to hydrolyze proteins. Enzymes are very specific (*i.e.*, pH, temperature, substrate) and can rely on many different factors to hydrolyze proteins (*i.e.*, enzyme/substrate specificity, enzyme/substrate concentration). This can result in a hydrolysate with a well-known chemical and nutritional composition (Tavano, 2013). This method of hydrolysis is the mildest reaction (*i.e.*, low temperatures, controlled pH, enzyme specificity) and leads to very little losses, yields a mix of amino acids, and polypeptides of varying lengths. This ability to control the reaction leads to a specific degree of hydrolysis; therefore, it can be correlated to the best-suited degree of hydrolysis for nutrition and functional properties. Therefore, it is less-likely that undesirable side reactions to occur, and fewer losses to take place (Sun, 2011). In addition to hydrolysis, enzymes can be used to induce other modifications such as crosslinking, and bridging. Enzymes such as transglutaminases, tyrosinases, and laccase can modify the function of proteins. Transglutaminases crosslink proteins via isopeptide linkages between a carboxyl group of one amino acid and the amino group of another amino acid. For example, Isaschar-Ovdat *et al.* (2015) investigated the use of tyrosinase in crosslinking soy Glycinin to stabilize oil-in-water emulsions, which improved the stability and formed gel like structures in the emulsion. Tyrosinase creates a cross-linked protein between the amino acids tyrosine and cysteine, lysine and tyrosine. Laccase forms tyrosine-tyrosine crosslinks, and disulfide bridges. Chen *et al.* (2010) investigated the use of laccase for stabilization of emulsions by cross-linking adsorbed beet pectin layers and determined that their use could be a way of incorporating soybean oil into more food products as the similar or better stability than uncoated droplets.

2.4 Enzymatic hydrolysis of a protein

Consumer benefits of consuming pea protein are extensive, however the food products containing pea protein are limited (Barac *et al.*, 2011). Challenges associated with pea protein are the lack of consumer acceptability, its bitter taste, and its limited native functionality (Barac *et al.*, 2011). Therefore, the employment of enzymes to modify pea protein is one strategy that may increase the use of pea protein in products by adding value. Currently, the hydrolyzed food proteins that dominate the market are casein, whey and soy (Sun, 2011). Enzymes are used to produce low allergenic milk products for babies, pet food, as well as high value food supplements. The purpose of hydrolyzed food proteins is to increase the nutrition and aid in the development of specialty

foods for children, the elderly and athletes by increasing the protein digestibility (Clemente, 2000). In addition, hydrolyzed food proteins are used to increase the functional properties of food products and increasing the acceptability by modifying the sensory characteristics (*i.e.*, removing undesirable odours, flavours) (Barac *et al.*, 2011). There are many methods of modification to achieve hydrolysis as mentioned above, however, enzymatic treatment produces a defined hydrolysate in a highly-controlled reaction through moderate temperature, enzyme substrate specificity, and specific pH for the enzyme to act. The hydrolysis reaction involves the addition of water to protein in combination with a protease, yielding the cleavage of a peptide bond. The extent of hydrolysis is defined by the degree of hydrolysis (%DH) which compares the number of peptide bonds cleaved, divided by the total number of peptide bonds (Adler-Nissen, 1984).

2.4.1 Enzyme classification

Proteases can be obtained from many origins and classified according to their sources: microbial and fungal biomass (*i.e.*, proteases obtained from *Aspergillus oryzae, Bacillus licheniformis, Bacillus amyloliquefaciens*), animals (*i.e.*, trypsin, pepsin) and plants (*i.e.*, papain from papaya, and bromelain from pineapple). Each enzyme obtained from different sources ranges in its specificity and its use in the food industry. Depending on their source, cost, and safety for food uses, the use of proteases is restricted. There are six main groupings of enzymes and are classified by their catalytic reaction. The protease enzymes are classified in class 3: Hydrolases and are classified by their catalytic structure of proteinases are based on the enzymes catalytic reaction; aspartic protease, metallo-protease, serine protease, cysteine protease, and mixed-catalytic type (Tavano, 2013). The enzyme mode of action can be further defined as either endopeptidase or exopeptidases. Endopeptidases cleave at the middle of the peptide, yielding a large polypeptide, while exopeptidases cleave at the end of polypeptide chains yielding a hydrolysate with di-/tri-peptides, or free amino acids (Panyam & Kilara, 1996). Exopeptidases are defined further by their cleaving location; carboxypeptidases cleave peptide bonds from the C-terminus of the protein, while amino peptidases cleave from the N-terminus (Panyam & Kilara, 1996). While enzymes are defined, and differentiated by their ability to cleave at certain locations, some enzymes called cathepsins can cleave at both C- and N-terminus. Cathepsins have both negative charges and positives charges that allow them to bind to the negative C-terminus

(cathepsin H), and positive N-terminus (cathepsins X) allowing cleavage at both ends of the protein (Tavano, 2013).

2.4.2 Changes to protein after hydrolysis

One of the major changes resulting from hydrolysis is the difference in taste. Consumers buy foods based on taste preferences, therefore a difference in taste could either enhance purchases, or decrease. When protein is hydrolyzed, bitter flavour components are released and considered a disadvantageous property associated when hydrolyzed protein are used for food (Humiski & Aluko, 2007). The most bitter-tasting hydrolysates are gelatin, meat, fish and pea ranging from highest to lowest. The released peptides may create the sense of bitterness due to the increase in hydrophobicity of the protein and degradation of the protein itself yielding bitter tastes (Adler-Nissen, 1984). The bitterness of protein can be controlled by selecting a protease that limits the amount of bitter and hydrophobic peptides released during hydrolysis based on their selectivity (Panyam & Kilara, 1996). For example, bitterness can be reduced if both endo-peptidases and exo-peptidases are combined. When peptides on the end of the protein chain are hydrophobic, exo-proteases cleave off the peptides and lose their bitterness. Neutral proteases (metalloproteases) generate less bitterness in hydrolyzed protein, compared to that of animal-based enzymes due to the specific cleavage site. Adler-Nissen (1984) investigated the effects of the control of the proteolytic reaction in attempt to reduce the bitterness of the hydrolyzed protein (*e.g.*, whey). The author found that a reduced degree of hydrolysis (DH = 3-5%) lowered the bitterness profile of the hydrolyzed protein; however, for the more extensively hydrolyzed protein, IEP following the hydrolysis reaction could reduce the bitter peptides. In addition, many functional properties change including; solubility, viscosity, emulsification, foaming and gelation. Meinlschmidt *et al.* (2016) conducted a sensory panel for soy protein hydrolysates treated individually with various enzymes (*i.e.*, Alcalase, pepsin, papain, Flavourzyme) and were judged based on their bitterness regarding the concentration of protein. The Alcalase hydrolysate had the highest bitterness, while the pepsin hydrolysate had sour and astringent taste properties, therefore the authors suggested the limitations in food products due to the tastes yielded. However, papain improved the sensory properties in comparison to the native protein while Flavourzyme hydrolysate sensory properties were like that of the native soy protein isolate. The authors suggested with these results that papain and Flavourzyme might potentially have good sensory application in food protein formulations.

The classification of enzymes and their individual preferences for cleavage site mentioned above greatly affects the structure of the protein. The main changes induced by enzymes on the native protein is the reduction of molecular weight, exposure of hydrophobic groups and increase of the number of ionizable groups (Tavano, 2013), (Panyam & Kilara, 1996). Enzymes allow the modification of the functional aspects of proteins (*i.e.*, solubility, gelation, emulsification, foaming, etc.) due to the change in native protein structure. The resulting changes in functional properties are outlined below and investigate how the changes in structure (*i.e.*, hydrophobicity exposure, hydrophilicity exposure, and size) affect the functionality.

2.4.3 Factors that influence hydrolysis

There are many factors that influence the degree of protein hydrolysis. Problems can arise due to hydrolysis (*i.e.*, bitter taste, off flavours, and slow reaction time), however the causes may depend on the enzyme used. Therefore, the optimal characteristics must be determined and utilized in practice to ensure the best quality and efficiency is utilized. Furthermore, the environmental characteristics (*i.e.*, temperature, pH, and substrate concentration) can impact the ability of the enzyme to hydrolyze. Enzymes have high specificity determined by the location that the enzyme can hydrolyze protein. Proteins have specific amino acid sequences, where the arrangement of residues defines the ability of the protein to bind with the substrate. Therefore, using a different enzyme on the same protein substrate can yield a hydrolysate with varying degree of hydrolysis and peptide length. Meinlschmidt *et al.* (2016) studied the effect of enzyme choice on the hydrolysis of soy protein isolate. The investigators found that enzyme choice had a major effect on the degree of hydrolysis. The highest degree of hydrolysis was obtained from Alcalase, while the lowest degree of hydrolysis was obtained from the enzyme PTN 3.0S from the same substrate. The authors attributed these differences in degree of hydrolysis from the individual specificity of enzymes.

The pH affects the shape of the enzyme as well as the shape of the substrate so that the enzyme may/may not bind to the active site and hydrolyze. Each enzyme has a pH optimum; however, the optimal pH is not the same for each enzyme. Protein's three-dimensional structure is defined by the amino acid sequence, where the tertiary structure forms a hydrophobic core of amino acids (*i.e.*, phenylalanine, and tyrosine) while acidic and basic amino acids are on the surface allowing hydrogen interactions to form. Therefore, a change in pH changes the

conformation of the enzyme and the protein structure either inhibiting the reaction from occurring, or allowing the reaction to proceed. Determining the pH optima can increase the efficiency of the hydrolysis reaction.

Temperature affects the degree of hydrolysis as enzymes have their own specific temperature optima. Karamac *et al.* (2002) investigated the use of trypsin in relation to temperatures on two different pea protein isolates during hydrolysis. The authors investigated four different temperature (35, 40, 45, and 50ºC) and determined that trypsin was most effective at 45ºC for *Pisane* pea protein isolate (Cosurca s.a., Momalle, Belgium), whereas 50ºC for *Propul*se pea protein isolate (Dutch Protein & Services, Tiel, The Netherlands), suggesting that in addition to different temperature, the substrate used can also influence what the optimal temperature is. The investigators also studied the influence of enzyme/substrate ratio on the hydrolysis of pea protein using trypsin. The lowest enzyme substrate ratio of 5 mAU/g (milliAnson units) for trypsin hydrolysis produced the lowest amount of hydrolysis in both pea protein varieties. The highest enzyme substrate ratio (35 mAU/g) produced the highest degree of hydrolysis on *Pisane* pea isolate, however, *Propulse* had the highest degree of hydrolysis with 15 mAU/gram for trypsin hydrolysis. These differences were attributed to the different composition of the pea protein, therefore limiting or enhancing pea protein. Time plays an important role in the hydrolysis reaction. The longer the enzyme remains active in solution, the higher the number of peptides cleaved. Therefore, it is important to consider the time needing to obtain a protein hydrolysate with a certain degree of hydrolysis and should be monitored. The functional properties of the protein hydrolysate depend upon the degree of hydrolysis, where the protein has been altered in various conformations depending on the enzyme used. Polanco-Lugo *et al.* (2014) investigated the structural properties of *Phaseolus lunatus* (Lima bean) protein isolate modified with pepsin-pancreatin. The authors hydrolyzed the protein isolate into two products based on the level of hydrolysis: ‘extensively hydrolyzed’ (DH>10%) and ‘limited hydrolyzed’ (DH<10%). The authors found that the limited hydrolysate had structural characteristics like that of the native protein isolate, however slight modification resulting from hydrolysis increased the hydrophobicity of the protein. The extensively hydrolyzed isolate was a mixture of polypeptides and peptides with low molecular weight. The authors noted that the limited hydrolyzed isolate had better functionality and could be incorporated into food products, while the extensively hydrolyzed

would be more beneficial as a therapeutic food. The authors attributed the differences in the structure based on the hydrolysis time with degree of hydrolysis (DH%).

The stability of the enzyme itself plays an important role in the ability to hydrolyze protein. The stability of the enzyme is based on its ability to withstand adverse conditions (*i.e.*, high temperatures, denaturants, and extreme pH) as well as preventing autolysis. Autolysis is common where the enzyme uses another enzyme as a substrate leading to auto digestion, yielding an inactive enzyme (Tavano, 2013). Therefore, depending on the enzyme used, the temperature and pH should be considered based on the enzymes ability to remain stable and active (Meinlschmidt *et al.,* 2016).

2.4.4 Nutritional impacts from hydrolysis

Hydrolysates have been used extensively in products for patients requiring a special diet, and additional nutritional support. These individuals requiring special formulations are unable to fully digest protein in its native structure therefore hydrolyzing protein is an alternative for those with Crohn's disease, malnutrition, phenylketonuria and other food allergies (Clemente, 2000). More specifically, enzymatic hydrolysis can yield defined amino acids with high quality. The decrease in molecular weight of the protein yields smaller peptides as well as free amino acids that can be readily digested in comparison to that of native protein. Exoproteases are most commonly used to manufacture food formulations for those needing additional nutritional support due to the free amino acid and smaller peptides. Hydrolysates are much better absorbed due to the higher amount of free amino acids, and tri/dipeptides compared to intact protein (Grimble, 1994). Nesse *et al.* (2011) studied the use of a fish protein hydrolysate in the formulation of a food product directed towards malnourished children. The authors determined that the fish protein hydrolysate obtained through endogenous enzyme hydrolysis could improve the body mass index (BMI) of malnourished children without causing adverse reactions. The results obtained were attributed to the hydrolyzed protein being more readily digested than the native protein.

Hydrolysis can yield bioactive peptides that have additional health-promoting effects in the human diet. Recently, pulses have been investigated for their bioactive peptide content with certain health promoting effects (*i.e.*, cancer-fighting properties, angiotensin converting enzyme, and anti-inflammation) (Lopez-Barrios *et al.*, 2014). A bioactive peptide is a certain amino acid sequence obtained from the hydrolysis of protein. Based on the source of protein, certain sequences can be

yielded. Legumes and pulses have been known for their high protein content in addition to their ability to aid in the prevention of certain illnesses (Lopez-Barrios *et al.*, 2014).

There have been many studies posed at eliminating the allergenicity of soy and other legume protein foods based on thermal, pressure, ultraviolet treatments and chemical modifications (Meinlschmidt *et al.*, 2016). However, the removal of allergens has not been fully successful. Hydrolysis can alleviate the allergenicity related to legume protein that can cause fatal results and increase risk factors for children with asthma. In addition, hydrolysis has been investigated to decrease the IgE reaction that food allergens cause in humans (Kasera *et al.*, 2015). Extensive hydrolysis produces hypoallergenic food products that result in small peptides ranging in size from 5000 to 500 Da (Panyam & Kilara, 1996). Kasera *et al.* (2015) studied the influence of enzymatic hydrolysis with Alcalase and Flavourzyme on the allergenicity of legume protein. The authors found a decrease in the allergenicity of legume protein due to the modification of peptides yielded from the hydrolysis reaction. The authors attributed the reduction in allergenicity due to the modified peptide being able to block the antigen-binding site. However, because of hydrolysis, the functional properties have been changed, and as mentioned above can lead to a change in taste (Meinlschmidt *et al.*, 2016). Clemente *et al.* (1999) studied the influence of enzymatic hydrolysis with Flavourzyme and Alcalase on chickpea protein isolate on the allergenicity. The authors found that the presence of 11*S* globulin chains that were hydrolyzed were responsible for IgE reactions obtained by exoprotease treatment, however the authors noted that with exo- and endoprotease treatment yielding an extensively hydrolyzed product had the highest reduction in allergenicity. The authors attributed the results to the presence of monovalent peptide sequences, which have lower allergenicity.

2.5 Modified protein functionality

2.5.1 Protein solubility

Having good protein solubility is a precursor for other functional attributes, such as emulsification, foaming and gelation. Solubility is defined as the concentration of protein that exists in the solvent at an equilibrium state, and relates to the balance of protein-solvent and protein-protein interactions (Kramer *et al.*, 2012). Both intrinsic (*e.g.*, protein structure, conformation, charge and hydrophobicity), extrinsic (*e.g.*, temperature, pH, different solvents and, the type and concentration of salts present) and processing (*e.g.*, enzymes, extrusion, fermentation,

extrusion, acid/base hydrolysis, shear and thermal treatments) factors can affect protein solubility as it can alter the level of protein denaturation and electrostatic forces which in turn impacts aggregation (Barac *et al.*, 2012; Kramer *et al.*, 2012). For the most part, proteins display a typical U-shaped pH-solubility profile where minimal solubility occurs at the isoelectric point (pI) where they display no net charge, and have higher solubility at both lower and higher pH values (Kramer *et al.*, 2012). At the pI, protein-protein aggregation is at its maximum, whereas at higher/lower pH values electrostatic repulsive forces between neighboring proteins keep the proteins dispersed in solution (Boye *et al.*, 2010).

Solubility of proteins also can be improved with higher temperature, until reaching its denaturation temperature. After which, proteins begin to unfold to exposed buried hydrophobic amino acids to promote a greater level of hydrophobic interactions between proteins and aggregate growth resulting in the loss of solubility (Perez-gago & Krochta, 2001). The addition of salts, and impact protein solubility differently depending on the type and concentration of salts present. For instance, the addition of a *salting-in* type salt (*e.g.*, sodium chloride) will enhance solubility of a protein as it acts to structure the hydration layer around the protein to promote increased protein-water interactions, however at a higher concentration NaCl will begin to have a negative effect as it will act to screen the electric double layer of the protein to promote aggregation and solubility loss (Jiang *et al.*, 2010; Kramer *et al.*, 2012). If a *salting-out* salt (*e.g.*, ammonium sulfate) is present, solubility will increase at very low concentrations, and then the proteins will precipitate out of solution based on their hydrophobicity (Kramer *et al.*, 2012). Ammonium sulfate promotes ion-water interactions, which disrupts the hydration layer surrounding the protein. As water molecules are pulled away from the protein, hydrophobic amino acids become exposed leading to greater protein-protein interactions (Jiang *et al.*, 2010).

Protein solubility can also be impacted through enzymatic modification with enzymes such as pepsin, papain, and Alcalase (Wu *et al.*, 1998; Zhao *et al.*, 2011; Cui *et al.*, 2013). These enzymes act to partially hydrolyze the protein's structure to decrease its molecular mass, release peptides and induce partial unfolding of its conformation to expose both buried hydrophobic and hydrophilic amino acids (Wu *et al.*, 1998; Cui *et al.*, 2013). These changes in structure can increase the protein's solubility, and expand its pH-solubility range (Zhao *et al.*, 2011). Cui *et al.* (2013) investigated the effect of pH (2-9) and limited pepsin hydrolysis (DH 2.5-7.5%) on the solubility of soybean proteins. The authors reported a solubility minimum at pH 4-5 (near its pI). Solubility

of the hydrolysates were slightly worse at pH 2 than the native SPI due to the exposure of hydrophobic groups, but was improved at the pI attributed to the release of low molecular weight peptides. At higher pH values (6-9), solubility was either improved or reduced relative to the native protein depending on the level of hydrolysis. Wu *et al.* (1998) found similar trends in solubility for SPI hydrolyzed with papain at different hydrolysis times (30-60 min) and pH 7. A typical U-shaped profile was evident with pH. Hydrolyzed SPI showed improved solubility over the native structure at pH values (3-7) attributed to the production of smaller molecular peptides produced by hydrolysis, as well as the exposure of polar amino acids that interact with water. Zhao *et al.* (2011) investigated the solubility of Alcalase hydrolyzed peanut protein (DH's 2.1%, 3.6% and 5.4%) in the pH range of 2-10. The U-shape curve was like the one found by Wu *et al.* (2010), with the lowest solubility occurring at pH 5 associated with the isoelectric point. The U-shaped curve changed with an increase in hydrolysis, where solubility improved near its pI (pH 5) relative to the native peanut protein with increasing levels of hydrolysis. The authors attributed the increase in solubility to the unfolded protein being able to adsorb water due to the now exposed hydrophilic groups and increased hydration of peanut protein suggesting that limited hydrolysis can be an effective method to increase protein solubility.

2.5.2 Emulsification

Emulsions are defined as mixtures of two or more immiscible liquids, where one phase is dispersed within the continuous phase of the other in the presence of mechanical shear and an emulsifier (*e.g.*, protein) (Wilde *et al.*, 2004; McClements, 2007). A protein's emulsifying abilities stem from their amphiphilic nature where both hydrophobic and hydrophilic amino acids are present at the surface, and their solubility and film forming capacity (McClements, 2007). However, since proteins are more hydrophilic in nature, they are only useful in stabilizing oil-in-water type emulsions (McClements, 2007). During emulsion formation, soluble proteins migrate towards the oil-water interface, where they unfold and re-align to position their hydrophobic amino acids towards the apolar oil phase and hydrophilic amino acids towards the polar aqueous phase. Protein aggregation then occurs until a viscoelastic interfacial film develops to stabilize the oil droplet. Chang *et al.* (2015) investigated the inter-relationships between the physicochemical, interfacial and emulsifying properties of pea, soy, lentil and canola protein isolates. The authors found that during the initial stage of emulsion formation, proteins require a high surface charge

and low hydrophobicity to effectively associate to the interface to lower interfacial tension. However, to form a good viscoelastic interfacial film, proteins are required to have a high surface charge and high hydrophobicity. Furthermore, the emulsifying properties of the protein ingredient are also related to the types of proteins present, their size, and their structure. For instance, Kimura *et al.* (2008) studied the emulsifying properties of 7*S* and 11*S* proteins from cowpea, fava bean, French beans and soy. The authors reported the 7*S* proteins had better emulsifying properties than the 11*S* proteins since their smaller size made them more soluble, and the lack of a stabilizing disulfide linkage allowed them to reorient better at the interface to form the emulsion. Therefore, selection of the most appropriate plant-based protein emulsifier should consider balancing protein properties needed to associate to the oil-water interface to that of forming a strong viscoelastic film.

Once the viscoelastic interfacial film is formed, the oil droplets take on properties of the protein in terms of their pH dependence. At pH values close to the isoelectric point (pI) of the protein, the oil droplet has no net charge and therefore promotes the flocculation and coalescence of droplets leading to emulsion instability, creaming and then phase separation (Damodaran, 2005; Can Karaca *et al.*, 2011). At pH values lower or higher than the pI of the protein, the oil droplet assumes a net-positive or -negative charge, respectively. Depending on the magnitude of the charge, oil droplets will repel once another through electrostatic repulsive forces to allow the droplets to be dispersed within the continuous phase of the emulsion (Can Karaca *et al.*, 2011). Furthermore, depending on the conformation and amino acid sequence, trains or loops of amino acids can extend from the oil droplet's surface to create steric stabilization of the emulsions (Damodaran, 2005). Additionally, proteins not absorbed to the oil-water interface also act to increase the viscosity of the continuous phase to resist droplet migration and creaming (Damodaran, 2005).

The application of enzymes (*e.g.*, papain, pepsin, trypsin and Alcalase) to induce the partial unfolding of the protein's conformation to expose buried reactive amino acids have been investigate for improving the emulsifying properties of plant proteins with both positive (Wu *et al.*, 1998; Polanco-Lugo *et al.*, 2014; Ghribi *et al.*, 2015) and negative outcomes (Zhao *et al.*, 2011; Avramenko *et al.*, 2012). Wu *et al.* (1998) investigated the effect of hydrolysis time using papain on the emulsifying properties of soy protein isolate. The authors found that papain modification significantly improved the emulsifying activity after 10, 30 and 60 min of hydrolysis. The best

emulsifying abilities took place after 60-min of hydrolysis due to the exposure of hydrophobic groups to the surface and formation of smaller protein particles. In the latter case, the smaller particles had improved solubility and had a greater surface area to partake in protein-lipid interactions.

Polanco-Lugo *et al.* (2014) also found an increase in emulsion formation when investigating the functional characteristics of lima bean protein isolate that was hydrolyzed with pepsin to produce a limited hydrolysate (DH >10%). The authors reported that emulsifying activity with limited hydrolysis was higher than that of the native protein isolate. The increase in activity was attributed to the increased solubility of the protein, as well as the increase in surface hydrophobicity after hydrolysis. Ghribi *et al.* (2015) investigated the emulsifying properties of chickpea protein isolate hydrolyzed with Alcalase to various degree of hydrolysis (DH 4.0%-8.6%). The investigators found that the emulsion activity index was increased at the lowest degree of hydrolysis (DH 4.0%) and negatively impacted with increased degree of hydrolysis (DH 8.6%). Emulsion activity increased as a result of higher solubility, and increase in surface hydrophobicity. The decreases were because of the inability of smaller peptides to interact at the oil and water interface which decreased the viscoelasticity. Therefore, the authors suggested that a low degree of hydrolysis was sufficient to increase emulsion activity. Tamm *et al.* (2016) also found that varying the degree of hydrolysis significantly affected the emulsion stability. The emulsion characteristics of native pea protein isolate to pea protein hydrolysate modified with Alcalase and trypsin to various degree of hydrolysis were investigated. The authors reported that the differences in stability of the emulsions were due to the DH values and enzyme used. Alcalase modified pea protein isolate could only form a stable emulsion with DH of 1.0%, while trypsin could stabilize emulsions at degree of hydrolysis to 6.0%. The authors suggest that this occurred due to the low specificity of Alcalase which negatively affected the functional characteristics while trypsin was more specific and suitable to form films with a denser interfacial film with higher viscoelasticity allowing more flexibility.

Avramenko *et al.* (2012) investigated the emulsifying properties of a lentil protein isolate that was partially-hydrolyzed with trypsin/heat to obtain degree of hydrolysis of 4, 9 and 20%. The authors reported that the emulsifying properties (*i.e.*, emulsification activity and stability indices) were poorer than the native protein, and that no additional loss in the emulsifying properties was observed with changes in the levels of hydrolysis between 4 and 20%. The authors hypothesize

the reduced emulsifying properties were due to the re-association of partially unfolded proteins that caused hydrophobic groups to become re-buried within the interior of the larger aggregate. Similarly, Zhao *et al.* (2011) found a negative impact of hydrolyzing protein on the emulsion ability of peanut protein isolate. Zhao *et al.* (2011) investigated hydrolyzed peanut protein isolate using Alcalase to varying degree of hydrolysis at 2.1, 3.6, and 5.4% as well as their emulsion characteristics. The hydrolysis could improve certain functional characteristics of peanut protein isolate such as solubility, but impaired the emulsifying activity index. The authors attributed the impairment in emulsion activity index to the decreasing surface hydrophobicity as hydrolysis increased. The decrease in the surface hydrophobicity may have deterred the ability of the protein to align at the oil-water interface resulting in impaired emulsion activity index. Mune Mune (2015) also found a negative impact on emulsion activity resulting from enzymatic hydrolysis of bambara bean protein concentrate. Mune Mune (2015) investigated the emulsifying activity of pancreatin modified bambara bean protein concentrate with varying hydrolysis conditions (*i.e.*, time, enzyme substrate ratio). The resulting hydrolysate lead to a significant decrease in emulsion activity. The authors suggested this occurred due to the smaller peptides formed from hydrolysis being unable to form a stable film around the oil droplets, which lead to coalescence because of formation of aggregates that inhibits the creation of the membrane on the oil-water interface.

2.5.3 Foaming

Foaming is an important functional characteristic in foods such as meringues and cakes as they contribute to both taste and textural aspects. Foams are a colloidal system containing small air bubbles dispersed throughout a continuous aqueous phase with application of shear and stabilized by an emulsified (*e.g.*, protein) (Damoradan, 2005). Foam formation and stabilization are dependent upon the structural characteristics of the protein (*i.e.*, molecular weight, flexibility, surface charge, and hydrophobicity) as well as the physicochemical characteristics (*i.e.*, solubility, isoelectric point). Foams are characterized by foaming capacity and their stability. Foam capacity is defined as the ability of a protein, under certain extrinsic conditions (*i.e.*, pH, temperature and ionic concentration) to form foam (Zayas, 1997). Intrinsic properties of the protein affecting the formation of foams include: protein flexibility, its isoelectric point, size and charge of the protein, and level of exposure hydrophobic groups (Zayas, 1997). Foam stability is the ability of the foam to withstand gravitational and mechanical stress that leads to decline of the foam volume as liquid

between the bubbles drains. Stability of foam is affected by the interfacial tension, continuous phase viscosity and film elasticity (Khalid & Elhardallou, 2015). However, the addition of a protein assists in the stabilizing the foam, in addition to increasing the foaming capacity.

Foams are generated by either super saturation, where gasses are dissolved in a liquid at increased pressure, or through the addition of mechanical forces (*e.g.*, homogenization and whipping) that disperses the air into the liquid to create foam. There are three significant stages in which protein foams are formed (Damoradan, 2005). Proteins diffuse to the interface, and orient themselves so that the hydrophilic amino acids are positioned towards the aqueous phase, and the hydrophobic amino acids towards the gas phase (*e.g.*, air). The denaturation of the protein in solution is influenced by the flexibility of the protein that ultimately reduces the interfacial tension, as well, influencing the ability for peptides to interact with each other though electrostatic, hydrophobic interactions and hydrogen bonds (Wilde, 2000; Damoradan, 2005). The rate of diffusion to the interface is greatly affected by the physicochemical properties of the protein due to the distribution of hydrophobic and hydrophilic residues on the protein surface.

The initial diffusion of the protein to the interface is largely determined by the surface hydrophobicity and anchoring to the interface, where the greater the surface hydrophobicity, the better the ability of the protein to foam (Barac *et al.*, 2011). In addition to hydrophobicity, flexibility is another important factor determining the interfacial properties of protein. Flexible proteins can unfold quickly at the interface, while less flexible films such as legume protein (*i.e.*, globulin) are slower at unfolding at the interface (Barac *et al.*, 2011). However, globular proteins contain more intramolecular bonds that stabilize the structure of the protein, and can greatly increase the stability of the foam (Wilde, 2000). Lastly, the polypeptides can form interfacial film through interactions, stabilizing the bubbles promoting foam formation. Even though globular proteins are slower at unfolding at the interface, they can form stronger intermolecular interactions stabilizing the interfacial film, and prevent coalescence (Wilde, 2000).

There are many factors affecting the stability of foam (*i.e.*, viscoelasticity of film, interfacial tension). The presence of an emulsifier can reduce the interfacial tension between the two phases and this can be achieved by the addition of a protein as a surfactant. Destabilization occurs due to the extent of surface tension, and gradient between the two. Coalescence of bubbles can occur due to the instability of film, leading to foam with less volume. The ability for legume proteins to foam is important for the development of plant-based protein foods. Plant proteins have

complex tertiary protein structure, which can be improved through physical and enzymatic modifications to alter the structure and increase the foaming properties (Damodaran, 2005). Authors reported limited hydrolysis (DH <10%) of plant based proteins with proteases (pepsin, trypsin, papain, Alcalase) can aid in the foaming capacity, while extensively hydrolyzed proteins (DH> 10%) can cause negative effects on the foaming capacity, and create a less stable foam due to the reduction in molecular weight resulting from hydrolysis (Govindaraju & Srinivas, 2006; Martinez-Villaluenga *et al.*, 2009; Zeng *et al.*, 2013; Polanco-Lugo *et al.*, 2014). Higher degree of hydrolysis yield a protein fraction with a lower molecular mass increasing the speed of diffusion to the interface to enhance foam formation (Barac *et al.*, 2011). However, small peptides may be formed that are too small to stabilize the interface. Therefore, extensively hydrolyzed protein can be expected to decrease foam stability due to fewer interactions between peptides (Barac *et al.* 2011).

Polanco-Lugo *et al.* (2014) hydrolyzed lima bean protein with pepsin and pancreatin to study the effect of degree of hydrolysis (1.7% and 16%) on the foaming properties and other functional characteristics. Proteins with limited hydrolysis (DH 1.7%) produced foam with greater stability near the isoelectric point compared to proteins that underwent extensive hydrolysis (DH 16%). The authors suggested that the smaller protein chains resulting from extensive hydrolysis are unable to form a flexible film to stabilize the foam. Similarly, Martinez-Villaluenga *et al.* (2009) found a decrease in foaming capacity with increased degree of hydrolysis for a soy protein hydrolysate modified with *Aspergillus oryzae* fungal protease. The authors found foam capacity to increase at low levels of hydrolysis (DH = 0.4%), whereas as degree of hydrolysis increased (>0.4%) foam capacity became reduced due to a higher level of small peptides that are unable to interact with each other to create a viscoelastic film to stabilizes the foam. Zeng *et al.* (2013) investigated the effect of degree of hydrolysis and enzyme substrate ratios (0.5%-4%) on the foaming properties of a soy protein isolate modified with papain, Alcalase and pancreatin. The authors found that the foaming capacity increased with all three enzyme treatments, where optimal foaming occurred at a DH of 1.6% for papain, 4.4% for Alcalase and 2.8% for pancreatin. However, as the DH values increased, foam formation was deterred due to the smaller sized proteins. The authors rationalized that papain might have been the optimal enzyme for foaming; as it does not have a broad specificity therefore cleaves far less locations on the protein. Govindaraju & Srinivas (2006) investigated the foaming properties of an arachin hydrolysate

modified with papain, Alcalase and fungal protease to achieve the desired degree of hydrolysis. The authors found that the Alcalase-modified protein with a low DH had better foaming properties relative those that underwent more hydrolysis

Extrinsic factors, such as pH also significantly affect the foam stability of hydrolyzed protein (Barac *et al.*, 2011; Wani *et al.*, 2015). Wani *et al.* (2015) studied the foaming properties of kidney bean hydrolysates modified with papain (DH 3.13%-8.63%) and found significantly higher foaming capacity compared to the native kidney bean isolate. As foaming capacity is related to solubility of the protein, the authors suggest that hydrolysis increased the foaming capacity due to the increase in solubility. The effect of pH on foam stability was also studied and found that hydrolyzed protein and native protein was most stable at pH 4 and 6. The authors attributed this to the pH being near the pI lessening the repulsive interactions and favoring protein-protein interactions and the protein forms a viscous film at the interface. Barac *et al.* (2011) studied the impact of enzymatic hydrolysis (DH 3.9-4.7%) with chymosin on the foaming properties of pea protein isolate at various pH values to find that the enzymatic treatment improved foaming capacity at low pH values, however had a negative effect on the foam stability at neutral/alkaline pH. The increase in the foam capacity of pea protein was attributed to the modification of molecular mass, and increased surface hydrophobicity, however the lack of stability may have been due to the protein film's permeability to gas.

2.5.4. Water holding capacity

Water holding capacity (WHC) refers to the ability of a protein to entrap or abide water against forces or processes to prevent losses in food (Zayas, 1997). WHC is a measure of the amount of water (g) that can be absorbed by one gram of protein (Zayas, 1997). The degree of protein-water interactions can have a big influence on its level of hydration and ability to become solubilized into solution and display other functional attributes. There are various types of water associated with the protein or within a protein matrix, including vicinal or constitutional water, multi-layer water or bulk (entrapped or free) water. Vicinal water is bound water that strongly interacts by water-ion and water-dipole interactions with the hydrophilic moieties on the protein's surface (Schnepf, 2013). Vicinal water forms the first single layer of water around the protein but may not cover hydrophobic sites, is un-freezable, and is strongly influenced by the intrinsic properties of the protein. Multi-layer water occupies the remaining sites (both hydrophilic and

hydrophobic) on the protein's surface with water-water, and water-protein hydrogen bonds, is largely un-freezable and adds several binding sites on the protein for water to further interact with (Schnepf, 2013). The bulk phase water interacts with the binding sites created by multilayer water and occupies the sites farthest away from the protein surface, is freezable and may be either free or entrapped within a protein matrix (Schnepf, 2013).

WHC can be influenced by both intrinsic and extrinsic factors. The intrinsic properties (*e.g.*, amino acid composition, size, conformation and isoelectric point) of the protein play a large role in the ability of protein to hold water. For instance, vicinal water binds to the hydrophilic amino acids on the protein's surface (Yin *et al.*, 2008). The size and conformation of the protein creates steric hindrances creating domains that inhibit water associations (*e.g.*, hydrophobic pocket) or entrap bulk water. Extrinsic factors, such as the presence of salts (may screen hydrophilic sites), the type of salts (water structuring or disordering salts – contributing to salting-in or -out, respectively), solvent pH in relation to the proteins isoelectric point (altering the amount of charge on the protein to abide water), and processing conditions (*e.g.*, thermal treatments, denaturing solvents/salts or enzymatic treatments) which induces changes to the intrinsic properties of the protein (Yin *et al.*, 2007; Cumby *et al.*, 2008; Guan *et al.*, 2008).

In terms of enzymatic hydrolysis, the proteins intrinsic properties are altered by cleaving and exposing more hydrophilic and hydrophobic groups to alter its surface properties, conformation and size. Cumby *et al.* (2008) studied the WHC of canola protein isolates treated with Alcalase and Flavourzyme with similar degree of hydrolysis (DH = 20.6, 18.9% respectively). The authors found that partial hydrolysis enhanced the WHC due to the release of small peptides (which abided more water) and since the protein became more hydrophilic (Cumby *et al.*, 2008). Yin *et al.* (2007) studied a decrease of WHC for hemp protein isolate hydrolyzed with trypsin with increasing levels of hydrolysis, which the authors attributed to decrease in exposed hydrophilic sites due to the aggregation of insoluble hydrolysates. Guan *et al.* (2008) investigated the effect of trypsin hydrolysis on the WHC of oat bran protein concentrates found the opposite effect where WHC improved with increasing levels of hydrolysis.

2.5.5 Oil holding capacity

The oil holding capacity of proteins is related to the ability of the protein associate with oil and to physically entrap it within its conformation or matrix. As such, surface area, and the level

of exposed hydrophobic moieties plays a big role in determining the level of oil a protein can hold (Zayas, 1997). By definition, OHC is a measure of the amount of oil (g) that can be absorbed by one gram of protein (Zayas, 1997). Many of the similar extrinsic and intrinsic properties discussed within the WHC section also apply here, where any form of protein unraveling or release of peptides/subunits during denaturation or hydrolysis that bring an increased number of hydrophobic groups to the surface or that increases its surface area will impact the protein's OHC (Periago *et al.*, 1998; del Mar Yust *et al.*, 2010; Mune Mune, 2015; Wani *et al.*, 2015). Furthermore, the method of protein isolate production can also impact OHC, as different methods select for different protein profiles (*i.e.*, different levels of globulins, albumins, prolamins and gliadins). For instance, Stone *et al.* (2015) compared the OHC values for pea protein isolate produced from different extraction methods. The authors reported that OHC was highest for salt extracted proteins followed by those prepared by micellar precipitation and isoelectric precipitation.

Mune Mune (2015) investigated the impact of degree of hydrolysis on OHC properties of cowpea flour hydrolyzed with pepsin to find enzymatic hydrolysis greatly improved OHC up to a maximum after 20-30% degree of hydrolysis. The authors attributed this increase to the change in protein conformation an increased number of hydrophobic groups exposed was observed. Periago *et al.* (1997) found a similar increase in the OHC with hydrolyzed pea flour using *Aspergillus satoi* protease. Wani *et al.* (2015) studied the OHC of kidney bean protein isolates hydrolyzed with papain to find values increased with hydrolysis time relative to the native protein. And del Mar Yust *et al.* (2010) investigated the OHC of chickpea protein isolates hydrolyzed with immobilized Alcalase to find improved OHC values relative to the native isolate. The authors also noted that a DH of 4.9% gave the best OHC values, while further degree of hydrolysis decreased the OHC. The authors attributed the decrease in OHC with increased DH due to exposure of ionic groups.

2.5.6 Gelation

Gels have structural significance in many food products as they control both quality and texture. Strong gels are continuous systems assembled from a solid phase, embedded in a solvent that forms a well-defined cohesive network that lacks fluidity and deformability (Panyam & Kilara, 1996; Banerjee & Bhattacharya 2012). In contrast, weak or fluid gels have similar structures except far fewer junction zones and have more deformability. Strong self-supporting gels are found used in hydrogel applications, gelled desserts and processed meats (*e.g.*, sausages),

whereas weaker non-self-supporting gels are found in applications such as salad dressings, yogurts and beverages (Banerjee & Bhattacharya, 2012). There are several critical points as a sol is converted into a gel. Initially, at the protein concentration increases, the protein solution goes from a dilute to semi-dilute sol at a critical concentration known as the 'critical overlap concentration' where significant chain overlap occurs leading initial aggregate formation and changes to solution viscosity. As the concentration increases further and aggregates continue to grow it undergoes a transition from a viscous solution to a viscoelastic fluid at the 'critical gelation concentration'. At this point, a three-dimensional continuous network extends through the medium and a weak gel is formed (Lamsal *et al.*, 2007). As protein levels increased further, the gel develops into a stronger network. A protein's gelling capacity is defined as the ability of a protein to form a three-dimensional network that displays solid-like viscoelastic behavior [*i.e.*, the dynamic storage modulus (G') is greater than the loss modulus (G'')] (Mession *et al.*, 2015). Protein gelation typically involves some level of protein denaturation upon heating, which induces unfolding of the quaternary and tertiary conformation of the protein to expose buried reactive sites. (Banerjee & Bhattacharya, 2012). Some level of aggregation then occurs via hydrophobic interactions and strengthened by the formation of disulfide bonding. The type of aggregate formed is dependent on the level of electrostatic repulsive forces present. If the amount of repulsion is low (*e.g.*, pH is near the protein's isoelectric point, or if high levels of salts were present), proteins aggregate randomly via cluster-cluster aggregation, whereas if the repulsion is higher (*i.e.*, pH away from the protein's isoelectric point or if low levels of salts were present) the proteins will align end-to-end to form fibrous-type (or 'strings of beads') aggregates (Renekema *et al.*, 2000). As the solvent cools, aggregation increases until for 3-D network forms. Upon further cooling, the gel network strength increases due to the formation of hydrogen bonding and non-covalent forces (*e.g.*, van der Waals forces and ionic bonding). Depending on the type of proteins present, the type and concentration of salts present, the magnitude and rate of temperature changes, solvent pH or the presence of other ingredients (*e.g.*, sucrose), protein aggregation, junction zone formation and gelation can be tailored to achieve different macroscopic properties.

For instance, Sun & Arntfield (2011) investigated the effect of heating and cooling rates (0.5, 1, 2 and 4 ºC/min) on the gelation properties of salt extracted pea proteins. The authors reported an optimal heating and cooling rate of 2 ºC/min and 0.5 ºC/min, respectively, allowed for the proper unfolding, rearrangement and alignment of proteins to form the strongest gel network

(*i.e.*, highest G′). Within legume proteins, 11*S* and 7*S* proteins display different gelling properties due to differences in molecular mass and level of bonding. The larger 11*S* proteins have six sub-units, with each subunit stabilized by disulfide bridging, making it more difficult to unfold. In contrast, the 7*S* trimer is comprised of three subunits stabilized by non-covalent forces and hydrophobic interactions therefore can unravel easier during the denaturation process. Thus, the ratio of 11*S* to 7*S* can impact the protein's gelling abilities. For instance, soy glycinin (11*S*) denatures at higher temperatures and forms stronger gels (due to stabilizing disulfide bonds) than soy beta-conglycinin (7*S*) which denatures at lower temperatures and forms weaker networks due to the lack of disulfide bonding (Utsumi *et al.*, 1997). O'Kane *et al.* (2004) investigated the gelation properties of pea legumin (11*S*) as a function of heating/cooling rates relative to that of soy glycinin. The researchers found that gel formation for pea legumin was unaffected by the heating rate, however with slowly cooling the legumin showed improved gel strength as it allowed greater time the disulfide linkages to form at higher temperatures. Relative to soy, gel networks formed with the 11*S* proteins were stronger.

Enzymatic hydrolysis can impact a protein's gelling behavior, where some level of unfolding can promote gelation through the exposure of previously buried reactive amino acid groups. In contrast, if proteins are hydrolyzed too much, the molecular size of the protein may decrease to a point that would prevent network formation. Zhao *et al.* (2011) investigated the heat-induced gelation properties of peanut protein isolate hydrolyzed with Alcalase to different degree of hydrolysis. The authors found good gel properties with 2.1% degree of hydrolysis. The investigators suggested that the increased exposure of sulfhydryl and disulfide bonds was higher than the native isolate where no disulfide bonds are present arachin (11*S* fraction). However, increasing the degree of hydrolysis inhibited gel formation due to decreased protein aggregation (Zhao *et al.*, 2011). Lamsal *et al.* (2007) studied the rheological properties of gels created from soy protein isolate hydrolyzed with bromelain to 2 and 4% degree of hydrolysis. The authors also reported that for the hydrolyzed samples, the gelation temperature and gel strength were reduced relative to the native protein. The authors attributed this decrease in gel strength was due to a reduction in hydrophobicity the amount of free disulfide groups required for gelation.

2.6 Bioactive compounds in pulses

There are several reasons why plant based proteins are under-utilized, as their nutritional conversions are not as efficient as meat resulting from the presence of heat-stable and -labile compounds that lead to detrimental health effects and limited utilization of protein or carbohydrate when consumed (Day, 2013). Pulse-based protein contains certain bioactive compounds (*i.e.*, phytate, protease inhibitors, lectins) that decrease the digestibility and conversion of protein into energy. These compounds can lead to digestive issues and inhibit the breakdown of protein. Therefore, peas can inhibit the nutrient uptake if the peas are not properly treated prior to consumption. Certain treatments such as heat and acid can degrade antinutritional factors and increase the bioavailability of protein. Processing methods such as fermentation, and enzymatic hydrolysis have been studied in increasing the digestibility and the nutritional value. In addition to antinutritional factors, pulses contain undesirable flavour profiles that makes them unappealing.

2.6.1 Total phenolics and condensed tannins

Total phenolics are composed of several different phenolic compounds (*i.e.*, flavonoids, condensed tannins, gallic acid, vanillin, proanthocyanidins, and phenolic acids), that are measured. Phenolic compounds have an astringency that potentially could be a result of the defense against the body against their antinutritional properties. Environmental growing conditions and plant genetics can impact the polyphenols present in the crop (Faller & Fialho, 2009). Polyphenolic compounds have at least two phenolic rings and are water soluble with molecular weights of 0.5 to 3 kDa. Phenolic compounds are biologically active, and research has been conducted to determine their disease preventative properties from cancer, diabetes, osteoporosis, and inflammatory disorders (Scalbert *et al.*, 2005; D'Archivio *et al.*, 2007). However, in the case of protein nutrition, total phenolics can impair protein digestion. Phenolic compounds can form complexes with protein molecules and when this occurs, the structure of protein changes leading to altered functional and nutritional properties (Ozdal *et al.*, 2013). The complexes of protein and phenolics create a more heat stable structure, and makes it less soluble. When proline rich proteins are present, polyphenolic compounds can interact and lead to the formation of an insoluble aggregate which can yield lower digestibility values (Soares *et al.,* 2011). Similarly, condensed tannins are thought to be part of the plants chemical defense against viruses, microbes, and herbivores. Tannins are polymeric flavonoids that are part of polyphenolics. In high tannin diets,

growth depression has been found and thought to occur from the decrease in the protein digestibility. Large proline rich proteins are able to bind with condensed tannins to a higher extent compared to small compact proteins resulting from the condensed tannins selectivity (Blytt *et al.*, 1988) In the presence of proteins, condensed tannins precipitate the protein, decreasing the solubility, and lead to complex formation with iron during digestion (Brune *et al.*, 1989; Diaz *et al.*, 2010). When protein precipitation occurs during digestion, the digestion of protein is decreased, as well as the availability for absorption leading to decreases in digestibility and can inhibit digestive enzymes. Therefore, the removal of polyphenolic compounds and condensed tannins could be beneficial to protein digestion.

2.6.2 Enzyme inhibitors

Enzyme inhibitors are compounds that inhibit the enzymatic reaction. In the case of protease inhibitors, digestive proteases that would act on the protein are inhibited leading to a reduction in proteolysis. Trypsin and chymotrypsin inhibitors are serine protease inhibitors, and when present in the diet can form stable complexes with the digestive enzymes leading to reduced activity. The protease inhibitors are categorized into two main groups: Kunitz and Bowman-Birk inhibitors which are based on their molecular weights and reactive inhibitory site. Kunitz type inhibitors (KI) are ~21 kDa and composed of 181 amino acids, containing two disulfide bonds. The reactive site of KI is located between residue 63 and 64. Comparatively, Bowman-Birk inhibitors (BBI) are smaller, ~7-9 kDa, and contain independent binding site for trypsin and chymotrypsin. However, BBI contain seven disulfide bonds and show stability against heat, acid, alkali and proteases such as pepsin. The mechanism of inhibition occurs similarly to when the protease binds with protein to hydrolyze peptide bonds; however, it binds with the enzyme inhibitor. After hydrolysis, the protease inhibitor is stabilized by the disulfide bond leading to inhibition of the enzyme. If proteases are inhibited because of protease inhibitors, there is a decrease in protein digestibility, making less available for absorption. Therefore, there is a need to remove these bioactive compounds to ensure adequate protein intake is achieved. Some methods such as boiling, soaking, and roasting have been used to inactivate these compounds. Rackis *et al.* (1986) found that autoclaving peas at 103 kPa for 15 min reduced pea, lentil, green gram, black gram, chickpea trypsin inhibitors to undetectable levels. Additionally, roasting reduced trypsin inhibitors in peas and green gram to undetectable levels. The high levels of heat associated with

autoclaving and roasting are thought to inactivate trypsin inhibitors because of protein denaturation. Shi *et al.* (2017) found that by soaking whole and split yellow and green peas, the trypsin and chymotrypsin inhibitors decreased. As enzyme inhibitors are low molecular weight proteins, they could potentially be solubilized in the solution and then removed along with the soaking liquid.

2.6.3. Other bioactive compounds

Pulses contain other bioactive compounds such as lectins, α-amylase inhibitors, saponins and phytic acid that could potentially lead to positive health effects, however can lead to reduced bioavailability of protein/starch digestion. Starch digestion can be interfered with when α-amylase inhibitors are present in the food ingredient. The enzyme inhibitor prevents the action of amylases during digestion, and therefore leaves the starch intact. Lectins are proteinaceous compounds that bind with carbohydrates, and have been found in chickpea and pigeon peas with less than toxic levels. Red blood cells contain glycoproteins that lectins can bind with and agglutinate that can result in damage to the gastrointestinal system and impair digestion. Moist heat can destroy lectins present in the pulses, therefore consumption of raw pulses could lead to detrimental health effects (Chavan *et al.,* 1986). Common methods of reducing heat labile anti-nutrients are heat treatments with cooking, or roasting, and non-heat labile method of reducing compounds include soaking, germination, alcohol wash treatment or fermentation. Phytic acid, or phytate, is known for their strong chelating ability with minerals such as phosphorous, iron, calcium and magnesium and zinc, which decreases their ability to be absorbed. A major concern of a diet high in phytate is iron deficiency. Phytate also can chelate with protein leading to decreases in protein bioavailability. Factors such as temperature, or fertilizer during the growing season can determine the phytate content, where cooler climate produce crops with less phytic acid, and high phosphate fertilizers produce crops with high phytic acid contents. Soaking, germination or fermentation has been found to decrease the phytic acid contents, which can activate the endogenous enzyme, phytase and reduce phytate to inositol and phosphate. The byproducts can lead to cancer prevention or hypocholesterolemia effect, as well as phytic acids ability to chelate minerals needed for cancers growth can deter cancers ability to reproduce (Zhour & Erdman, 1995). Saponins are a non-heat labile compound, consisting of a sugar moiety, can bind with cholesterol making the molecules too large to be absorbed. However, saponins have hemolytic activities and can lead to detrimental

health effects (Jood *et al.*, 1986). Saponins can be reduced by soaking in a solution of minerals, therefore domestic processing can reduce their levels before consumption (Jood *et al.*, 1986).

2.7 Protein quality and bioavailability of pulses

Protein quality is dependent upon the nutritional composition of amino acids which depending on the source of protein can be complete (*i.e.*, contains all essential amino acids) or incomplete (*i.e.*, lacking one or more essential amino acids). In addition to their nutritional composition, the bioavailability of proteins and the body's ability to utilize the protein from food plays an important role in the quality. The source of the protein is an important factor to consider, as the amino acid composition is dependent on the source. Pulses are high in lysine, leucine, aspartic acid, glutamic acid and arginine, however lacking in methionine, cysteine and tryptophan. Therefore, pulses are a complimentary protein to cereal crops (*i.e.*, lacking in lysine, and methionine) (Pulse Canada, 2016). Furthermore, certain sequences of native protein in pulses resist digestion due to the tertiary structure that protects the cleavage of peptide bonds. Heat and acid treatments open the protein up into a more exposed form and the bonds can be made more available for digestion. In addition, enzymatic hydrolysis treatment can similarly open the protein up, and increase the ability for digestive enzymes to access and absorb protein.

Protein Digestibility Corrected Amino Acids Score (PDCAAS) is a simple method that is internationally accepted for quality assessment of protein introduced by the FAO/WHO in 1991 (FAO/WHO, 1991; Sarwar, 1997). PDCAAS measures protein based on the amino acid score which is the ratio of the limiting amino acid in your protein, in relation to a reference amino acid pattern of a 3-5 year-old (*i.e.*, most energetically demanding age), and protein digestibility. The PDCAAS method does not consider proteins with extra nutritional value, as proteins cannot score more than 100% in comparison to the reference protein (Sarwar, 1997). Furthermore, PDCAAS does not account for the aforementioned bioactive compounds and the potential adverse reactions that may occur due to their presence in food (Sarwar, 1997). PDCAAS also utilizes fecal protein content, which can cause problems as microbial protein can influence the amount of protein measured. The Digestible Indispensable Amino Acid Score (DIAAS) method is another internationally approved method, where in this method the dietary protein quality represents the amount of amino acids absorbed at the end of the small intestine. This gives a better idea of the amino acids absorbed, as it does not use the gastrointestinal microbial protein that can yield a

higher result – using ilium-digestibility instead. DIAAS can be used for diets of mixed protein sources, does not truncate the protein scores, and provides regulatory information to classify food products.

Enzymatic hydrolysis can aid in increasing the protein quality scores in the aforementioned methods. This can occur due to the cleavage of proteins, enhancing the ability for absorption and digestion. This is especially important for plant-based proteins, as certain sequences are unable to be digested in the body, and therefore are not fully utilized. Clemente *et al.* (1999) investigated the protein quality of chickpea protein hydrolysates hydrolyzed with Alcalase and Flavourzyme. The authors found that extensively hydrolyzed (DH > 50%) chickpea protein isolate had adequate amount of essential amino acids, however significant losses of phenylalanine and arginine occurred. In addition, the authors completed an *in vitro* digestibility of the hydrolysates, and found that they comparable to the starting material. Peragio *et al.* (1998) investigated the nutritional properties of pea flour hydrolyzed with a protease obtained from *Aspergillus satoi.* The authors found that the resulting amino acid profile of the hydrolyzed pea flour was slightly modified, where increases in isoleucine, leucine, lysine, cysteine, phenylalanine, threonine, alanine, arginine and aspartic acid. In addition, the addition of the enzyme was found to decrease the trypsin inhibitor, as well as phytic acid content, increasing the bioavailability of protein.

3 MATERIALS AND METHODS

3.1 Materials

Air-classified pea protein-enriched flour (pea protein-enriched flour; PPEF) was kindly donated by Parrheim Foods (Saskatoon, SK) for this research. Enzymes used in this study for hydrolyzing PPEF include: (1) trypsin solution from porcine pancreas; (2) pepsin from porcine gastric mucosa; (3) protease from *Aspergillus oryzae* (Flavourzyme*)*; (4) protease from *Bacillus* sp. (Savinase); (5) protease from *Bacillus amyloliquefaciens* (Neutrase); (6) crude papain; and (7) protease from *Bacillus licheniformis* (Alcalase). All chemicals and enzymes used in this study were purchased from Sigma-Aldrich Co. (Oakville, ON), and were of reagent grade.

3.2 Enzymatic hydrolysis

Optimization of the hydrolysis process

Initially, a 1% (w/w) protein solution was prepared by dispersing 1 g of PPEF in 100 g in an appropriate buffer and pH associated with each of the 7 enzymes outlined in Table 3.1. The solution was magnetically stirred at 4°C overnight. The solution was then brought to room temperature, pH readjusted and transferred to a dark reaction beaker to protect the reaction from light and placed into a shaking water bath at 37°C or 45°C depending on the enzyme used (Table 3.1). The solution was allowed to shake for 1 h prior to adding the enzyme to allow it to acclimatize. An enzyme solution was prepared at a ratio of 1 g of enzyme to 250 g of protein (or 0.004 g of enzyme per 1 g of protein). Initially, the enzymatic reaction was carried out at an enzyme/substrate ratio of [1/250] over a 120-min period, with samples (0.25 mL) being taken after 5, 10, 20. 30, 40, 60, 80 and 120 min for determination of the degree of hydrolysis using the TNBS (2,4,6-trinitrobenzenesulfonic acid) method (Adler-Nissen, 1979). TNBS reacts with primary amino groups to form a chromophore which can be measured spectrophotometrically. Depending on the number of primary amino groups in the solution, representing cleaved portions of the protein, the greater the absorbance reading. First, a control sample was taken before the enzyme

was added (*i.e.*, the time 0 sample). The enzyme was then added to the solution, and protein-hydrolyzed samples were taken thereafter. The samples (control at 0 min, and hydrolyzed sample at 5, 10, 20, 30, 40, 60, 80 and 120 min) were comprised of 0.25 mL 1% (w/w) protein sample. Samples were then added to 2 mL of 1% (w/v) sodium dodecyl sulfate (SDS) in buffer and heated at 80°C for 10 min to deactivate the enzyme. The tubes were then left to cool to room temperature (~30 min). This was followed by the transfer of 0.25 mL of the protein and SDS buffer solution into a second set of tubes containing 2 mL of buffer used for the enzyme reaction. The mixture was then vortexed for 10 s to ensure thorough dispersion. A volume of 2 mL 0.01% TNBS was added, vortexed for 10 s, and placed in a covered water bath for 1 h at 50°C. To terminate the TNBS reaction, 4 mL of 0.1 N HCl was added, and the tubes were left to cool to room temperature (~10 min) before being read at 340 nm using a UV-VIS spectrophotometer (Genesys 10S, Thermofisher Madison, WI, USA). glycine standard curve was then prepared to equate the degree of hydrolysis to the hydrolyzed samples. In brief, a 1.5 mM glycine stock solution was made using 0.0028 g of glycine in 25 mL of Milli-Q water. From this stock solution, a range of glycine concentrations was prepared through dilution with buffer to obtain levels of 0, 0.03, 0.06, 0.08, 0.1 and 0.3 mM. For instance, the 0 mM (blank) contained 2.25 mL of buffer, the 0.03 mM concentration was made using 90 µL of 1.5 mM glycine solution, and 2,160 µL buffer, and 0.06 mM concentration was made using 120 µL of 1.5 mM glycine solution and 2,130 µL buffer and so on.

To determine the degree of hydrolysis, the total acid hydrolysis was completed. For this, PPEF (0.024 g protein basis) was mixed with 7.5 mL of Milli-Q water for 30 min at room temperature, and then an additional 7.5 mL of 6 N HCl was added and a tight-fitting screw cap lid was applied to the tube. The mixture was then heated at 110°C for 20 h using a forced air oven to complete the total acid hydrolysis. Upon completion, the solution was filtered using a Whatman No. 3 filter paper (GE Healthcare UK Limited, Buckinghamshire, UK). The solution's pH was then adjusted to pH 7.0 using 1 N NaOH. Then 5 mL of the filtered solution were diluted with 5 mL of Milli-Q water. The TNBS reaction was used to obtain an absorbance reading, as previously described. Total acid hydrolysis was measured in triplicate.

Based on the preliminary results, the manufacturer's pH and temperature conditions proved to be optimal within the recommended buffer for PPEF (data not shown) and showed fast rates of hydrolysis using trypsin, papain, Savinase, and pepsin. However, Flavourzyme, Neutrase and

Alcalase were not very effective at modifying PPEF (data not shown, therefore was removed from further use). To control degree of hydrolysis to reach degree of hydrolysis values of 2-4% and 10-12%, the E/S ratios of [1/1000], [1/2500] and [1/5000] for the four enzymes at the recommended pH and temperatures were tested. Based on this, conditions were selected to have controlled hydrolysis to obtain degree of hydrolysis between 2-4% and 10-12% (Table 3.2 & Figure 3.1).

Table 3.1. Properties of commercial enzymes used to partially-hydrolyze PPEF.

Enzyme	**pH**	**Buffer**	**Temp. (ºC)**	**Specificity of cleavage**
Trypsin	6.8, 7.8*,8.8	5 mM Sodium phosphate	30ºC, 37ºC*	Serine protease
Pepsin	2.6*, 3.0, 4.0	5 mM Citric acid	25ºC*, 37ºC	Broad preference
Papain	5.2, 6.2*, 7.2	5 mM Sodium phosphate	37ºC, 67ºC*	Cysteine protease
Protease from *A. oryzae* (Flavourzyme)	5.5, 6.5, 7.5*	5 mM Sodium phosphate	37ºC*	Both endo and exopeptidase
Protease from *Bacillus sp.* (Savinase)	6.5, 5.5*, 7.5	5 mM Sodium phosphate	37ºC*, 30ºC	Serine protease
Protease from *B. licheniformis* (Alcalase)	6.5, 7.5*, 8.5	5 mM Sodium phosphate	37ºC*	Serine protease - endopeptidase
Protease from *B. amyloliquefaciens* (Neutrase)	5.2, 6.2*, 7.2,	5 mM Sodium phosphate	37ºC*	Serine protease-endopeptidase

Notation (*) refers to optimal conditions recommended by the supplier.

Table 3.2. Enzymatic conditions identified for enzymatically-treating PPEF with to obtain degree of hydrolysis (DH).

	pH	Temperature	[E/S] ratio[1]	Time	%DH
Trypsin	7.5	37°C	[1/5000]	20 min	2.3
				70 min	10.0
Pepsin	2.6	37°C	[1/2500]	30 min	2.3
				70 min	10.4
Papain	6.2	45°C	[1/2500]	20 min	4.0
				40 min	11.3
Savinase	7.5	37°C	[1/5000]	30 min	2.1
				70 min	10.0

[1][E/S] ratio refers to the grams of enzyme per gram of protein.

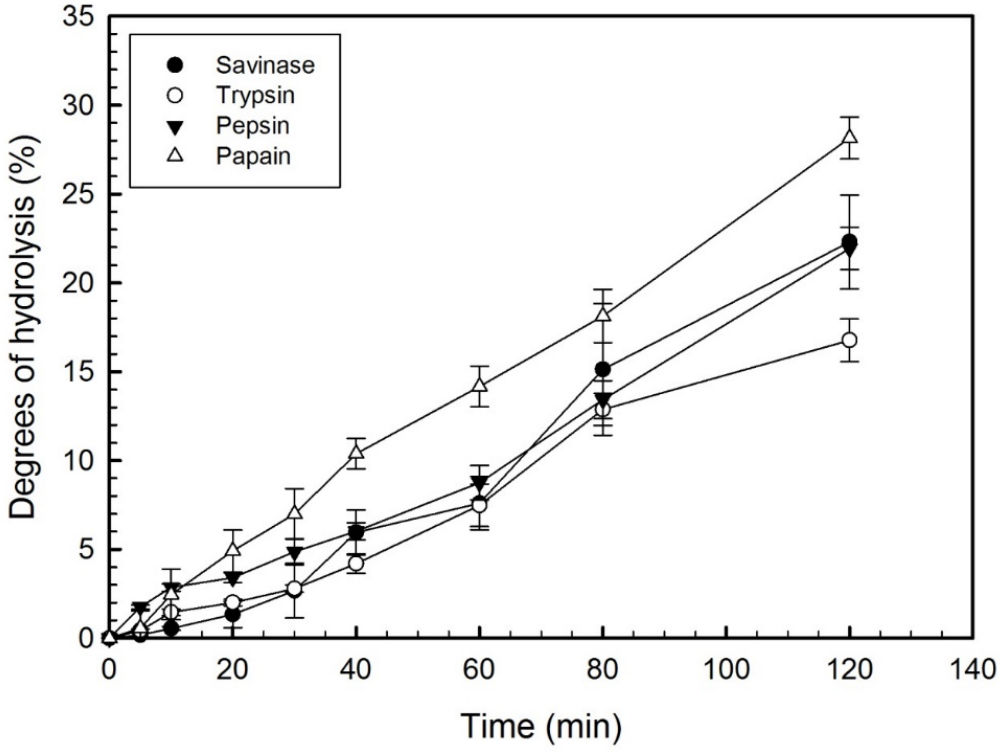

Figure 3.1. Degree of hydrolysis of PPEF using TNBS method versus time using four enzymes under conditions shown in Table 3.2. Error bars represents the mean ± one standard deviation (n=3)

Scaling-up the enzymatic reaction

In order to obtain enough sample for experiments associated with the physicochemical, nutritional and functional properties, a large amount PPEF was hydrolyzed, requiring a batch scale-up of 50-times. The scaled-up process involved dispersing 50 g of PPEF in 5,000 g (*i.e.*, 1% w/w) of buffer within a 5-gallon vessel for the four enzymes (papain, pepsin, trypsin and Savinase). The buffer was pH adjusted and allowed to magnetically stir overnight at 4°C. The following day, the pH of the solution was re-adjusted, and left to warm to room temperature (~1 h). The solution was then added to a fermentation tank (BioFlo III Batch/Continuous Fermenter, New Brunswick Scientific, NJ, USA) and warmed to the respective temperature specific for the enzyme. After 1 h of heating, the enzyme was added and left to hydrolyze based on times given in Table 2.2. To terminate the reaction 10 mL of 4 M HCl was added, followed by a subsequent heating step for 30 min in an 80°C water bath, where the 5-L hydrolyzed protein solution was divided into 2-L beakers. The solution was then left to cool to room temperature before pH adjustment to 4.6 to induce protein precipitation. A pellet was collected by centrifugation (Sorvall RC5C Plus Superspeed centrifuge. Asheville, NC, USA) at 7,148 × *g* for 30 min. The supernatant was drained off, and the pellet was re-suspended with pH 8.5 Milli-Q water to make the pH of the solution pH 7.0. The slurry was frozen at -30°C, and then freeze dried. The freeze-dried powders were then ground using a coffee grinder into a fine powder and then stored at 4°C until further analysis.

To determine the extent of protein hydrolysis, and changes to the size of protein an SDS-PAGE was performed using the method of Laemmli (1970) using a 12% separating gel at pH 8.6 ad 4% stacking gel at pH 6.8. Protein samples, 1% (w/w) in water, were left to stir overnight and the following morning, 50 µL of solution were then dispersed in 50 µL of 2× SDS-PAGE sample buffer containing 20 mM Tris-HCl buffer at pH 7.6, 10% SDS solution, 2% β-mercaptoethanol, 50% (v/v) glycerol and 0.01% bromophenol blue, and then heated for 10 min at 85ºC, followed by centrifugation at 7,500 × *g* for 10 min. Subsequently, the gel was stained with 0.25% Coomassie blue stain for 1 h, followed by de-staining with de-ionized water overnight. The protein bands were then captured as digital images, where images are used to estimate the molecular weight determination against a set of standards. Protein bands were quantified using ImageJ® (National Institutes of Health Bethesda, Maryland, USA). The protein bands were measured via volume, where volume is determined by the sum of pixel intensity for all pixels in each section.

3.3 Proximate composition

The proximate composition of unheated and treated PPEF samples (moisture, protein, lipid and ash) from the scaled-up batch were determined according to AOAC methods: 925.10, 997.09 (N% x 6.25), 920.85 and 923.03 respectively (AOAC, 2005). All analyses were performed in triplicate and reported on a moisture-free basis. The methods used are briefly described below.

Moisture

Moisture contents of unheated and treated PPEF samples were obtained gravimetrically using a gravity-flow convection oven (Fisher Scientific Isotemp Standard Lab Oven, Thermo Fisher Scientific Inc., Waltham, MA, USA) set to 105ºC, according to Official Method 925.10 of AOAC International (AOAC, 2005). Aluminum dishes (57 mm) were pre-dried in the oven for 1 h and placed in a desiccator to cool to room temperature. Samples of 1.0 g were weighed into the pre-dried dishes and dried overnight. The following day, the samples were removed from the oven and cooled to room temperature for one hour in a desiccator. They were then weighed on an analytical balance. Moisture Content (%MC) was calculated using the following equation (Eq. 3.1):

$$\text{Moisture (\%)} = \frac{\text{weight of sample} - \text{wt of dried sample}}{\text{wt of sample}} \text{x } 100\% \qquad \text{[Eq.3. 1]}$$

Ash

Ash contents of unheated and treated PPEF samples was measured gravimetrically using a muffle furnace (Fisher Scientific Isotemp Basic Muffle Furnace, Thermo Fisher Scientific Inc., Waltham, MA, USA) according to a modification of Official method 923.03 of AOAC International (AOAC, 2005). In preparation, porcelain crucibles were pre-dried for 1 h at 525ºC, and then cooled for 2 h before transfer into a desiccator to cool to room temperature. Added to pre-dried crucibles were 0.5 g of samples, following a step to pre-ash by charring on a hot plate set to high heat in a fume hood until fully blackened. The samples were then placed in the muffle furnace and heated overnight at 525ºC to obtain a white ash. Sample crucibles were cooled for 2 h before

transfer to a desiccator and allowed to cool to room temperature. Ash content is calculated with the following formula (Eq. 3.2):

$$\text{Ash}(\%) = \frac{\text{weight of sample} - \text{weight of dried sample}}{\text{weight of sample}} \text{x } 100\% \qquad \text{[Eq. 3.2]}$$

Lipid

Lipid contents of unheated and treated PPEF samples were measured using the Goldfisch apparatus according to the modification of Official method 920.85 of AOAC International (AOAC, 2005). In preparation, glass beakers were pre-dried in a forced air oven at 105ºC for 1 h, taken and placed into a glass desiccator and left to cool for 1 h to room temperature. Samples of 0.6 g were weighed and added into cellulose extraction thimbles and placed in the extraction sleeve. Glass beaker vessels were weighed and recorded and then filled with ~40 mL of petroleum ether. The beakers were then placed on the Goldfisch apparatus and the heat started. The samples were left to extract for 6 h to allow for sufficient removal of lipid components from the sample. The petroleum ether was left to evaporate, then the beakers were placed on a heating plate at 250ºC for 30 min, followed by placing them in a forced air oven overnight. The beakers were then placed into a glass desiccator to cool, and then re-weighed. The lipid % was calculated by using Eq. 3.3:

$$\text{Lipid } (\%) = \frac{\text{weight beaker after} - \text{weight beaker before}}{\text{sample weight}} * 100\% \qquad \text{[Eq. 3.3]}$$

Protein

Protein content of unheated and treated PPEF samples were determined using the LECO method according to the official method 997.09 of AOAC International methods (AOAC, 2005) using a FP628 LECO analyzer (3000 Lakeview Avenue, Saint Joseph, MI) Approximately 0.250 g of samples was weighed into a sample chamber followed by combustion of the sample at 1000ºC in the presence of oxygen. The carbon and the nitrogen in the sample are subsequently converted to CO_2 and NO_x. The gasses were separated by chromatography and measured in a thermal conductivity cell. The protein content was determined using the conversion factor of 6.25.

3.4 Physicochemical properties

Surface charge

The surface charge of the unheated and treated PPEF samples was measured at pH 7.0 based on the method of Avramenko *et al.* (2013). Protein solutions of 0.0625% (w/w) were prepared using 10 mM sodium phosphate, pH 7.0 buffer, and stirred overnight at room temperature. The solutions were then re-adjusted to pH 7.0 before measurement. The surface charge was measured at 25°C using a Zetasizer Nano-ZS90 instrument (Malvern Instruments, Westborough, MA). The electrophoretic mobility (U_E) of the protein were used to calculate the zeta potential (ζ; units: mV) by applying the Henry equation (Eq. 3.4):

$$U_E = \frac{2\varepsilon \cdot \zeta \cdot f(\kappa\alpha)}{3\eta} \qquad \text{[Eq. 3.4]}$$

where ε is the permittivity (units: F (Farad)/m), $f(\kappa\alpha)$ is a function related to the ratio of particle radius (α; units: nm) and the Debye length (κ: $units; nm^{-1}$), $and\ \eta$ is the dispersion viscosity (uunits: mPas). The Smoluchowski approximation $f(\kappa\alpha)$ was set as 1.5, as is accustom for folded capillary cells and, with particle larger than 0.2 μm dispersed in moderate electrolyte concentration (>1mM). The Smoluchowski approximation assumes that: a) concentration of particles (protein) is sufficiently-high such that such thickness of the electric double layer (Debye length) is small relative to the particle size ($\kappa\alpha \gg 1$), and b) ζ is linearly related to U_E. All measurements are reported as the mean± one standard deviation (n=3).

Surface hydrophobicity

Surface hydrophobicity of unheated and treated PPEF samples was determined based on the methods of Kato & Nakai (1980) using the fluorescent probe 8-anilino-1-naphthalenesulfonic acid (ANS). Protein solution of 0.025 % (w/w) were dissolved in pH 7.0 10 mM sodium phosphate buffer were stirred overnight at 4°C overnight (~16 h). The following day, the pH was adjusted to pH 7.0, followed by dilutions of the stock solution to create protein concentrations of 0.005%, 0.010%, 0.015%, and 0.020% with pH 7.0, 10 mM sodium phosphate buffer. To prepare sample solutions, 1.6 mL of each protein concentration was taken and 20 μL of 8 mM ANS probe solution, then vortexed for 10 s and kept in the dark for 5 min. The fluorescence intensity was measured

with FluoroMac-4 spectrofluorometer (Horiba Jobin Yvon Inc, Edison, NJ, USA) with excitation and emission wavelengths set at 390 and 470 nm, respectively, with slit widths at 1 nm for both excitation and emission. A protein blank solution was prepared by taking the readings of a 1.6-mL solution of each protein concentration added with 20 µL of pH 7.0 10 mM sodium phosphate buffer at the same wavelengths as mentioned above. Fluorescence intensity values for the ANS blank and protein blanks were subtracted from the fluorescence intensity against protein solutions containing ANS. The initial slope (S_o), of the plot of the fluorescence intensity against protein concentration was calculated by linear regression analysis and used as an index of the protein surface hydrophobicity ($S_o - ANS$).

3.5 Functional Properties

Water holding capacity (WHC) and Oil holding capacity (OHC)

The water holding (WHC) and oil holding (OHC) capacities of unheated and treated PPEF samples were measured according to of Yin *et al.* (2008) with slight modifications. For WHC, 0.5 g of PPEF samples and 10 mL of distilled deionized water were added to 10-mL capped centrifuge tubes and vortexed for 10 s every 5 min for 10 min. The protein solution was left to stand for 3 h at room temperature and then vortexed for 30 s, every 5 min for 30 min and then centrifuged at 3,000 × g for 30 min. The supernatant was drained, and the mass of the resulting pellet was weighed. The amount of water absorbed was determined based on differences in mass prior and after the test of the PPEF sample (reported as g water /g protein). A similar method is used with OHC, except the water was replaced with canola oil. The supernatant was drained, and the mass of the resulting pellet was measured and reported as g oil/ g protein. All measurements were made in triplicate.

Solubility

The solubility of unheated and treated PPEF samples were tested based on the methods of Achouri *et al.* (2005) and Barac *et al.* (2015). Protein samples of 100 mg were dispersed in 10 mL of Milli-Q water, followed by pH adjustment to 4.0, 7.0 and 10.0 using 1 N HCl or 1 N NaOH. The protein solutions were stirred for 30 min at room temperature, followed by centrifugation at 965 × g for 30 min. The supernatant was extracted and used to determine the amount of protein using the method of Bradford (1976). A standard curve was created using bovine serum albumin

(BSA) by making a standard curve of 0, 25, 50, 75, 100, 125, 250 and 500 µg/mL using Milli-Q water as a blank. To make the sample, 30 µL of the standard solution or the unknown protein concentration sample was taken and 1.5 mL of Coomassie reagent were added to a 2-mL centrifuge tube and vortexed for 10 s to mix well. The absorbance was then measured using a UV-VIS spectrophotometer at 595 nm. The solubility was calculated as the percent ratio of protein in the supernatant to that of the total protein in the initial sample. All measurements were made in triplicate.

Foaming capacity (FC) and stability (FS)

The foaming capacity and stability of unheated and treated PPEF samples were measured based on the method of Wilde & Clark (1996). In preparation, a 1% (w/w) protein was prepared and pH adjusted to 4.0, 7.0 or 10.0 using 1 N NaOH or HCl. The solutions were then left to stir overnight for promotion of protein solubility. The following day, the solutions were re-adjusted to pH 4.0, 7.0 or 10.0 prior to foam analysis. To prepare the foam, 15 mL of the adjusted solution was transferred into 400 mL beakers and homogenized using an Omni Macro Homogenizer (Omni International, Marietta, GA, USA) with a saw tooth probe at speed 4 for 5 min. The position of the saw tooth probe was immersed in the protein solution just below the surface to generate the foam. Following 5 min of foam preparation, the sample was transferred to a 50 mL graduated cylinder and the volume of the foam immediately after transfer was recorded as V_1. The foam was left to sit, undisturbed on the counter for 30 min, and the volume of the foam was measured and recorded as V_2. When recording, the upper level of the foam as well as the lower level of the foam was recorded. The foaming capacity and stability was calculated using Eq. 3.5 and 3.6:

$$\%FC = \frac{V_1}{15\ mL\ initial\ volume} * 100 \qquad \text{[Eq. 3.5]}$$

where V_1 is the volume observed immediately after homogenization.

$$\%FS = \frac{V_2 - V_1}{V_1} * 100 \qquad \text{[Eq. 3.6]}$$

where V_2 is the volume observed after 30 min of static storage at room temperature, and V_1 is the volume observed immediately after homogenization. All measurements were made in triplicate.

Emulsifying activity (EAI) and stability (ESI) indices

The emulsifying properties were determined for unheated and treated PPEF samples based on the method of Pearce & Kinsella (1978). In brief, a 0.25% (w/w) protein solution was prepared at pH 4.0, 7.0 and 10.0 and left to stir overnight at room temperature. The following day, the pH of the solutions was readjusted. Five grams of the 0.25% protein solution and 4.0 g of canola oil were measured into a 50-mL centrifuge tube, and homogenized using an Omni Macro Homogenizer (Omni International, Marietta, GA, USA) with a 20 mm saw tooth probe at speed 4 (~7,200 rpm) for 5 min. Immediately after homogenization, 50 µL of the emulsion was taken from the bottom of the tube (0 min) and added to 7.5 mL of 0.1% sodium dodecyl sulfate (SDS). After 10 min of static storage at room temperature, another 50 µL sample was taken from the bottom of the tube. The sample absorbance was then measured at 500 nm using a Genesys 10 UV-VIS spectrophotometer (Thermo Scientific, Madison, WI, USA) using a plastic cuvette (1 cm path length). The ESI and EAI were calculated using Eq. 3.7 and 3.8:

$$EAI\ (m^2) = 2T\left(A_0 * \frac{dilution\ factor}{C} * \phi * 10000\right) \quad \text{[Eq. 3.7]}$$

where T=2.303, A_0 is the absorbance immediately after emulsion formation, dilution factor is 151, C is the weight of the protein per unit volume of aqueous phase before emulsion formation (g/mL) and ϕ is the oil volume fraction of the emulsion.

$$ESI\ (\text{min}) = A_0 * \Delta t/\Delta A \quad \text{[Eq. 3.8]}$$

where A_0 is the absorbance immediately after emulsion formation, Δt is the change in time between 0 and 10 min and ΔA is the change in absorbance of the emulsion ($A_0 - A_{10}$). All measurements were made in triplicate.

3.6 Nutritional properties

Bioactive compounds

(a) Total phenolic content. The total phenolic content was determined using the Folin-Ciocalteu assay by Singleton and Rossi (1965). Phenolics were extracted using 5 mL of 1% HCl in methanol to extract 1 g of unheated and treated PPEF samples. This extraction procedure was initially placed on a rotating shaker for 2 h, then centrifuged at 1,050 × *g* for 10 min. This extraction was repeated twice more, however for 20 min of extraction. The supernatants were pooled for use in the Folin-Ciocalteu assay. A 1-mL aliquot of the pooled extract was added to a 25-mL volumetric flask containing 9 mL of distilled water. The blank was prepared using distilled water instead of sample extract. Folin-Ciocalteu phenol reagent (1 mL) was added into the mixture and shaken. The mixture was allowed to stand for 5 min before the addition of 10 mL of 7% Na_2CO_3solution. The volume was then made up with distilled water. The samples were incubated at room temperature for 90 min and then the absorbance of the samples was read at 550 nm against the blank using a UV-VIS spectrophotometer. A standard curve was prepared using gallic acid (100, 200, 300, 400, and 500 µg/mL). Total phenolic content was expressed as mg of gallic acid equivalents (GAE) per 100 grams of sample.

(b) Condensed tannins. Condensed tannin analysis was performed based on the Vanillin method of Price *et al.* (1978). A working vanillin reagent was prepared by mixing a stock solution of 1% vanillin in methanol and 8% HCl in methanol at a 1:1 volume ratio. A standard curve was performed daily making serial dilutions using a catechin solution made with 0.3 mg of catechin per mL of absolute methanol. 0, 0.1, 0.2, 0.3, 0.4 and 0.5 mL of the catechin solution was pipetted into a set of five tubes and the volume was adjusted to 1 mL with methanol, with a second set of tubes prepared the same way. The two sets of tubes were incubated in a 30ºC water bath. After incubation for 10 min, 5.0 mL of the working vanillin reagent was added to the first set of tubes, while 5.0 mL of 4% HCl was added to the second set of tubes. The addition of solution to each set of tubes was performed in 1 min intervals. The two sets of tubes were incubated for exactly 20 min, then the absorbance was read using a spectrophotometer (Thermofisher Scientific spectrophotometer, Madison, WI, USA) at 500 nm. The standard curve was made by plotting the absorbance vs. mg catechin, and the equation of the line was obtained. Protein samples of 0.2 g was extracted using 10 mL of absolute methanol for 20 min. After extraction, the samples were

centrifuged at 3,000 × g for 10 min (VWR Clinical centrifuge, VWR International, Mississauga, ON). After centrifugation, 1 mL of the supernatant obtained was transferred to 3 tubes, where two tubes were reacted with 5 mL of working vanillin reagent, while the third was incubated with 5 mL of 4% HCl for 20 min followed by absorbance reading. Levels of condensed tannins were determined according to Eq. 3.9:

$$\text{Tannins (mg of catechin equivalent per mL of extract)} = Abs_1 - Abs_2 - \left(\frac{b}{\alpha}\right) * 2{,}000\ mg\ of\ sample/10\ mL\ of\ extract \quad \text{[Eq. 3.9]}$$

(c) Trypsin inhibitor activity. Trypsin inhibitor activity was determined using the AACC method 22-40.01. Samples were prepared by weighing 0.01 g of unheated, heated and enzymatically treated PPEF and then extracted with 25 mL of 0.01-N NaOH for 3 h. The slurry was centrifuged at 3000 × g for 20 min at 4ºC. Following centrifugation, the pH of the supernatant was adjusted to pH 9.0 using 1 N HCl. The adjusted supernatant was then pipetted into five 15-mL test tubes at the following volumes: 0.0, 0.6, 1.0, 1.4 and 1.8 mL, then adjusted to 2.0 mL with distilled water. The tubes were incubated at 37ºC in a water bath with 2 mL of pre-warmed trypsin solution for 10 min. After exactly 10 min, 5 mL of pre-warmed Na-benzoyl-D, L-arginine 4-nitroanilide hydrochloride (DL-BAPNA) substrate solution at 37°C was added to the sample tubes. The tubes were incubated for another 10 min before termination of the reaction by the addition of 1 mL of 30% acetic acid. The solutions were filtered through Whatman No. 2 paper. The absorbance of the filtered solutions were determined at 410 nm using a UV-VIS spectrophotometer. DL-BAPNA is a chromogenic substrate that complexes with active trypsin and can be absorbed at 410 nm (Aviles-Gaxiola *et al.*, 2017). The TIU content of the samples was calculated using Eq. 3.10:

$$\text{TIU content} = \frac{\text{TIU}}{\text{mg sample}} = \frac{\text{TIU}}{\text{mL of extract taken}} \times \frac{\text{25 mL of extract}}{\text{500 mg of sample}} \times \text{D} \times \frac{100\%}{100\% - \text{MC}} \quad \text{[Eq. 3.10]}$$

(d) Chymotrypsin inhibitor activity. The chymotrypsin inhibitor activity was determined based on the method of Makkar *et al.* (2007), which measures the concentration of the chymotrypsin enzyme using casein as a substrate on the unheated, heated and enzyme treated PPEF. The enzyme solution was prepared at a concentration of 40 µg/mL. To prepare the enzyme solution, 4 mg of chymotrypsin was dissolved in ~80 mL of 0.001 M HCl with 0.08 M $CaCl_2$. A 1% casein solution was prepared by dissolving 1 g casein in 80 mL of 0.1 M pH 7.6 borate buffer, and dissolved by heating on a heat plate and gradually adjusting the pH to 7.6 to facilitate dissolving of casein which is relatively insoluble. The casein solution was then brought to 100 mL volume after being completely dissolved and then prewarmed 1 h prior to analysis. Tricholoroacetic acid (TCA reagent) was prepared by dissolving 18 g TCA, 18 g anhydrous sodium acetate and 20 mL glacial acetic acid, and brought to 1 L volume. Initially, 1 g of protein sample was extracted using 10 mL of pH 7.6, 0.1 M borate buffer by vortexing for 1 min, then put on a rotating shaker for 1 h, then centrifuged at 3,000 × *g* for 10 min at 4°C. The following volumes were pipetted into a set of glass test tubes: 0.0, 0.2. 0.4, 0.6 and 0.8 mL in duplicate, and diluted to 1 mL volume with 0.1 M pH 7.6 borate buffer. To both sets of tubes, 1 mL of chymotrypsin solution was added and left to incubate for 10 min at 37°C. The first set of tubes served as a blank, therefore 6 mL TCA reagent was added, followed by the addition of 2 mL of casein. The second set of tubes is the working set, therefore, only the 2 mL of casein was added and left to incubate for 10 min, followed by the addition of 6 mL of TCA reagent. The solutions were then left to sit for a for 30 min at temperature (21°C), followed by filtration step using Whatman no. 2 filter paper (GE Healthcare UK Limited, Buckinghamshire, UK). The absorbance of the filtered solution was then read at 275 nm using a glass cuvette. Chymotrypsin inhibitor activity was calculated using Eq 3.11:

$$\text{CIU content}=\frac{\text{CIU}}{\text{mg sample}}=\frac{\text{CIU}}{\text{mL of extract taken}}\times\frac{\text{10 mL of extract}}{\text{1000 mg of sample}}\times \text{DF}\times\frac{100\%}{100\%-\text{MC}}$$

[Eq. 3.11]

where CIU is the absorbance of the sample subtracted from the blank, which is subsequently divided by the amount of mL of extract taken in each individual tube and DF is the dilution factor of 10.

Protein quality

(a) Amino acid analysis. The amino acid composition of untreated, unheated, heated and enzymatically treated pea protein concentrate were determined using a Pico-tag^{TM} amino acid analysis system and high-performance liquid chromatography (HPLC). The determination of 15 amino acids was performed according to the method of Bidlingmeyer *et al.* (1987). Initially, ~20 mg protein was prepared and mixed with 15 mL of 6-N HCl in screw-cap Pyrex tubes, followed by flushing the tubes with N_2. The tubes were fitted with a cap and incubated at 110ºC for 20 h to hydrolyze the protein into individual amino acids for HPLC separation and determination. Tryptophan was determined following the AOAC method 988.15 (2005) with slight modification. Protein samples were hydrolyzed using 10 M NaOH and incubated for 20 min in a boiling water bath followed by incubation in a 110ºC oven for 16 h followed by HPLC determination using reverse phase liquid chromatography with UV detection to determine tryptophan. The concentrations of sulfur containing amino acids, methionine and cysteine, were determined following AOAC method 985.28 (2005) using ion-exchange chromatography with modification. The cold performic acids was used for cysteine and methionine oxidation and they were kept for reaction at 4ºC overnight. The sulfur containing amino acids were oxidized with performic acid and hydrolyzed with 6 M HCl at 110ºC for 18 h.

(b) Amino acid score. The determination of the amino acid score is measured by the ratio of 1 g of target protein to the reference protein. The amino acid composition of reference protein was determined using the specifications of amino acid requirements for children 2-5 years (amino acid, mg/g protein): histidine, 19; isoleucine, 28; leucine, 66; lysine, 58; methionine + cysteine, 25; phenylalanine + tyrosine, 63; threonine, 34; tryptophan, 11; valine, 35 (FAO, 1991). The amino acid score characterizes the most limiting essential amino acid.

(c) In vitro *protein digestibility.* The *in vitro* protein digestibility was determined based on the method of Tinus *et al.* (2012) using the pH drop method which measures the change in pH resulting from the protein solution being digested by a multi-enzyme solution. The multienzyme solution was made fresh each day by mixing 31 mg of chymotrypsin, 16 mg trypsin, and 13 mg of protease type XIV from *Streptomyces griseus.* The pH of the multienzyme solution was adjusted to pH 8.0 ± 0.05 with 0.1 M NaOH or HCl. A protein solution of 62.5 ± 0.5 mg of protein was added to 10 mL of MilliQ water. The protein solution was left to stir in a pre-heated water bath set to 37°C for 1 h. The pH of the protein solution was adjusted to 8.0 ± 0.05 with 0.1 M NaOH and

HCl prior to the addition of 1 mL of the enzyme mixture that was previously mentioned. Following the addition of the enzyme solution to the protein solution, the pH of the protein solution was recorded every 30 s for 10 min. The *in vitro* protein digestibility was calculated using Eq. 3.12:

$$IVPD = 65.66 + 18.10 * \Delta\ pH_{10\ min} \quad \text{[Eq. 3.12]}$$

where the $\Delta\ pH_{10\ min}$ is the change in pH from the initial time 0 min to 10 min.

(d) In vitro *Protein Digestibility Corrected Amino Acid Score (IV-PDCAAS).* The IV-PDCAAS was calculated as the product of the amino acid score and *in vitro* protein digestibility.

3.7 Statistical Analysis

Statistical analysis was completed using SigmaStat 4.0 (San Jose, CA, USA). An individual degree orthogonal contrast analysis was performed with the two- and three-way analysis of variance (ANOVA) to test pre-determined questions of untreated pea protein and enzyme hydrolyzed pea protein. For example [1] untreated vs. hydrolyzed; [2] enzyme type: trypsin, Savinase, papain, and pepsin; [3] degree of hydrolysis: 2-4% vs. 10-12%; [4] enzyme × degree of hydrolysis; and for certain functional properties tested at various pH: [5] pH: 4.0, 7.0, and 10.0; [6] pH × degree of hydrolysis; [7] enzyme × pH and; [8] enzyme × degree of hydrolysis × pH. Significant difference was alpha (α)<0.05.

4 RESULTS AND DISCUSSION

4.1 Surface and functional properties of enzymatically-modified air-classified pea protein-enriched flour treated by different enzymes to varying levels of hydrolysis

4.1.1 Composition

The lipid, protein and ash contents of various treated and untreated pea protein-enriched flours (PPEF) are summarized in Table 4.1.1 as a function of the enzyme-type and degree of hydrolysis (DH 2-4% vs 10-12%). Samples tested include: a) untreated, which was unaltered from the original commercial air-classified PPEF; b) unheated PPEF, which was stirred overnight at the corresponding pH used for enzymatic hydrolysis (pH control); c) heated PPEF, which followed the same pH/time/temperature course as the enzymatically hydrolyzed treatments but without the enzymes (temperature control); and d) enzymatically-hydrolyzed PPEF, which was hydrolyzed to different times to obtain different two different levels of degree of hydrolysis (2-4% vs. 10-12%). The treated samples (unheated, heated and hydrolyzed) underwent a centrifugation step to collect the protein for analysis, while the untreated sample did not. An individual degree of contrast analysis found that protein, lipid and ash levels in the hydrolyzed PPEF (regardless of enzyme-type and degree of hydrolysis) were significantly different than the untreated samples ($p<0.001$) (Table 4.1.2). The two controls (associated with pH and temperature) were not included in the statistical analysis. Overall, protein levels were found to have increased concentration from 49.3% to 53.8% from with hydrolysis, whereas lipid levels decreased from ~4.3% to ~3.7%, and ash levels decreased from ~7.0% to 2.4% (Table 4.1.1). It is hypothesized that the changes in levels is due to the hydrolysis of some protein, which disrupted protein-lipid complexes while in solution. These liberated lipids, along with solubilized minerals and carbohydrates were lost in the supernatant after centrifugation, leading to their reduction in the final dried powder. This preparation process then resulted in increased protein levels. A similar concentration of protein, lipid and ash in the protein rich fraction of air-classified peas were found by Sosulski & Youngs

(1979). Protein, lipid and ash contents were determined to be 52.4%, 3.3% and 7.4%, respectively in the air-classified pea samples.

Table 4.1.1 The proximate composition of air-classified pea protein-enriched flour (PPEF) enzymatically-modified with different enzymes to achieve different degree of hydrolysis (DH = ~2-4% vs. ~10-12%)[1]

Treatment	Protein (%, d.b.)	Lipid (%, d.b.)	Ash (%, d.b.)
Untreated PPEF	49.3 ± 0.1	4.3 ± 0.0	7.0 ± 0.4
Trypsin (T) and Savinase (S)			
• Unheated (control)	54.4 ± 0.2	3.1 ± 0.4	2.1 ± 0.2
• Heated (control)	54.4 ± 0.0	3.8 ± 0.1	2.1 ± 0.1
• T (DH 2.3)	52.0 ± 0.0	4.2 ± 0.0	2.0 ± 0.0
• T (DH 10.0%)	54.6 ± 0.4	3.3 ± 0.1	2.6 ± 0.3
• S (DH 2.3%)	53.7 ± 0.3	5.3 ± 0.1	1.8 ± 0.0
• S (DH 10.4%)	56.4 ± 0.2	3.8 ± 0.2	2.5 ± 0.1
Papain (Pa)			
• Unheated (control)	54.6 ± 0.4	4.9 ± 0.9	2.5 ± 0.1
• Heated (control)	54.3 ± 0.1	5.0 ± 0.6	1.9 ± 0.1
• Pa (DH 4.0%)	53.5 ± 0.1	4.0 ± 0.1	1.8 ± 0.1
• Pa (DH 11.3%)	54.5 ± 0.7	3.1 ± 0.6	2.5 ± 0.1
Pepsin (Pe)			
• Unheated (control)	54.7 ± 0.0	3.5 ± 0.4	2.2 ± 0.2
• Heated (control)	54.3 ± 0.5	3.7 ± 0.4	2.2 ± 0.2
• Pe (DH 2.1%)	52.4 ± 0.8	3.7 ± 0.2	2.5 ± 0.1
• Pe (DH 10.0%)	51.1 ± 0.1	2.5 ± 0.3	3.1 ± 0.5

[1]Enzyme treatments are as follows: a) trypsin and Savinase (pH 7.5, 37°C, 20 min, and 70 min); b) papain (pH 6.2, 45°C, 20 min and 40 min); and c) pepsin (pH 2.6, 37°C, 30 min and 70 min). All enzyme reactions were terminated by heating to 85°C for 30 min for trypsin, Savinase and pepsin, while papain was heated to 100°C for 30 min. Controls included: a) unheated (at pH values corresponding to those used during enzymatic treatments, but without heating) and b) heated (heated under the same temp. and pH conditions as the enzymatic treatments, but without enzymes). Upon completion all enzymatic treatments, the pea protein-enriched flour was pH adjusted to pH 7.0 prior to drying of the ingredient into a powder. All data is reported as the mean ± one standard deviation.

Table 4.1.2. Individual degree of freedom (orthogonal) contrast analysis using the general linear model of the composition, surface and functional properties of untreated and enzymatically-hydrolyzed pea protein-enriched flours.

	Composition			Surface properties		Functional properties						
	Protein	**Ash**	**Lipid**	**Charge**	**Hydro-phobicity**	**Sol**	**WHC**	**OHC**	**EAI**	**ESI**	**FC**	**FS**
Main effects												
Untreated vs. Hydrolyzed	p<0.001	p<0.001	p<0.001	p<0.01	p<0.001	p<0.001	p<0.001	p<0.001	p<0.01	p<0.001	p<0.001	p<0.001
Enzyme	p<0.05	p<0.05	p<0.05	p<0.01	p<0.001	p<0.01	p<0.001	NS	p<0.001	NS	p<0.001	p<0.05
DH	p<0.05	p<0.001	p<0.001	p<0.05	p<0.001	p<0.05	p<0.001	p<0.001	NS	p<0.01	NS	NS
pH	-	-	-	-	-	p<0.001	-	-	NS	p<0.001	p<0.001	p<0.001
Interactions												
Enzyme-type x DH	p<0.01	NS	NS	NS	p<0.001	NS	NS	NS	NS	NS	NS	NS
pH x DH	-	-	-	-	-	NS	-	-	NS	NS	p<0.001	NS
Enzyme x pH	-	-	-	-	-	p<0.05	-	-	p<0.001	NS	p<0.001	NS
Enzyme x DH x pH	-	-	-	-	-	NS	-	-	NS	NS	p<0.001	NS

Conditions:

Enzyme-type (trypsin, Savinase, papain, and pepsin)
pH (4.0, 7.0 and 10.0)
Degree of hydrolysis (DH) (2-4% and 10-12%)

Abbreviations:

Sol (solubility), WHC (water hydration capacity), OHC (oil holding capacity, EAI (emulsion activity index), ESI (Emulsion stability index), FC (foaming capacity), FS (foaming capacity), (-) (Not applicable) and NS (Not significant)

An individual degree of freedom contrast analysis found the interaction between enzyme-type and degree of hydrolysis to be significant for protein ($p<0.05$), but not for ash and lipid ($p>0.05$) (Table 4.1.2). Because of that, both enzyme-type and degree of hydrolysis will be discussed separately for ash and lipid. In the case of proteins, enzymatic treatment with both trypsin and Savinase was found to increase the protein levels from 52.0 to 54.6% as the degree of hydrolysis increased from 2-4 and 10-12%, respectively (Table 4.1.1). In contrast, protein levels for PPEF treated with papain or pepsin remained independent of the degree of hydrolysis, with average protein levels of 53.9 and 51.8%, respectively (Table 4.1.1). Protein contents of hydrolyzed wheat gluten varied based on the enzyme used in a study by Kong *et al.* (2006). The authors reported lower protein levels were found in pepsin treated samples (78.3%), while pancreatin- and trypsin-treatment had higher protein contents (80.2% and 80.9%, respectively), suggesting that enzyme type is a significant determinant of protein contents. As the degree of hydrolysis increased from 2-4% to 10-12% (regardless of the enzyme-type), the lipid levels were found decrease from 4.3% to 3.2%, whereas the ash levels were found to increase from 2.0% to 2.6%, respectively ($p<0.001$) (Table 4.1.2). In the case of enzyme-type (regardless of DH), lipid levels were found to be 4.2%, 4.0%, 3.6% and 5.3% for trypsin-, papain-, pepsin-, and Savinase-treated PPEF, respectively ($p<0.05$) (Table 4.1.2). The higher levels of proteins and ash may be the result of a greater amount of lipid liberated from protein-lipid complexes during hydrolysis by the serine proteases (trypsin and Savinase) relative to the cysteine-type (papain) and acidic-type (pepsin) based proteases. This lipid would then have been removed during the centrifugation step of preparation resulting in increased amounts of both ash and protein, and lower amounts of lipid in the powder after drying. Similar increases in protein and decreases in lipid and ash with increasing Alcalase hydrolysis were observed by Cai *et al.* (2013) for pine nut protein isolate. The initial protein, lipid and ash content was 87.2% and increased to ~94.0%, 0.9% and 4.4%, respectively, after 25% DH. Comparable results to Cai *et al.* were observed by Ghribi *et al.* (2015). In that study, chickpea protein isolate was hydrolyzed with Alcalase and the authors found that protein content increased from 78.5% in the untreated sample to 79.2% at 4% DH. Protein content increased further to 83.7% at 14.7% DH. Ghribi *et al.* (2015) determined the increase in protein was a result of proteolytic action, increasing the soluble protein. This was in combination with a decrease in fat content from 4.5% in the untreated sample to 2.2–1.1% after 14.7% DH. The

decrease in lipid contents may be beneficial for long-term stability of food by decreasing the effects of oxidation.

Sodium dodecyl polyacrylamide gel electrophoresis

PPEF is composed of various proteins, and polypeptide bands that have been used to identify proteins in pea are legumin (60 kDa) and vicilin (~50 kDa for α-, β-, and γ-fractions) (Tzitzikas *et al.*, 2006). The SDS-PAGE of various treated and untreated pea protein-enriched flour (PPEF) were conducted to analyze the changes in sizes (*i.e.*, primary structure). The modification to the protein by the hydrolysis was observed through the changes in molecular weight bands in the SDS-PAGE gel (Figure 4.1.1), and were quantified through densitometry using ImageJ software (Figure 4.1.2; Table 4.1.3). The untreated PPEF shows a higher concentration of large molecular weight peptides (~93 to ~17 kDa) and a lower amount of smaller molecular weight peptides (~11 to ~8 kDa), shown in Table 4.1.3. The untreated sample had a higher concentration of larger molecular weight protein bands as there was no modification to alter the structure of protein. The unheated and heated control had similar banding patterns and were not affected by the pH or the heat treatment of the protein. Guan *et al.* (2007) also found that pH and heat does not affect the concentration or banding pattern of hydrolyzed oat bran protein concentrate. For the enzyme treated PPEF (Lanes 4, 5, 6, 7, 10, 11, 14 and 15) generally showed decreases in larger molecular weight peptides and increases in concentrations at lower molecular weight peptides (Table 4.1.3). Pepsin has a broad specificity and preferentially cleaves peptides with aromatic or carboxylic L-amino acids. Pepsin cleaves at C-terminal to Phe+Leu and to a lesser extent Glu linkages, however it does not cleave at Val, Ala and Gly linkages. Pepsin-treated samples (lanes 14 and 15) show a reduction in the ~42 kDa band and an increase in the ~29 kDa band relative to the other hydrolyzed samples. Pepsin-treated samples (2-4% and 10-12% DH) in lanes 13 and 14 appear to have more of a breakdown in molecular weights bands from ~20 kDa to ~5 kDa. The changes in protein sizes were not well-defined suggesting that pepsin treatment has broad specificity (*i.e.*, unspecific hydrolysis) and leads to various low molecular weight peptides. Papain also has a broad substrate specificity, and can hydrolyze at bulky hydrophobic or aromatic amino groups. Papain-treated samples (2-4% and 10-12% DH) in lanes 10 and 11 did not have a specific band that decreases more than the other, showing that it has more of a random attack than the other enzymes. Trypsin and Savinase are serine proteases with similar conditions used for hydrolysis.

Trypsin is the most specific of the enzymes used and cleaves at Lys and Arg preferentially, while Savinase cleaves at hydrophobic residues, however they can hydrolyze many others. More specifically, trypsin-treated samples (2-4% and 10-12% DH) in lanes 4 and 5 appear to have lower density at the ~65 kDa bands than the other hydrolyzed samples. One of the main differences of trypsin, is the ability to breakdown the 70 kDa band (4.3% at 2-4% DH, and 2.5% at 10-12% DH), whereas the other enzymes did not change from 7.0-7.8%. Savinase-treated samples (2-4% and 10-12% DH) in lanes 6, and 7 reduce the ~42 kDa band more than the other hydrolyzed samples. Hydrolyzed samples have altered quaternary and tertiary structure, which results from the cleavage of peptides from the protein chain, or from the aggregated protein to yield smaller peptides or subunits (Avramenko *et al.*, 2013). Little differences between the bands could not be visually-discerned without using image analysis for quantitative analyses of the gels.

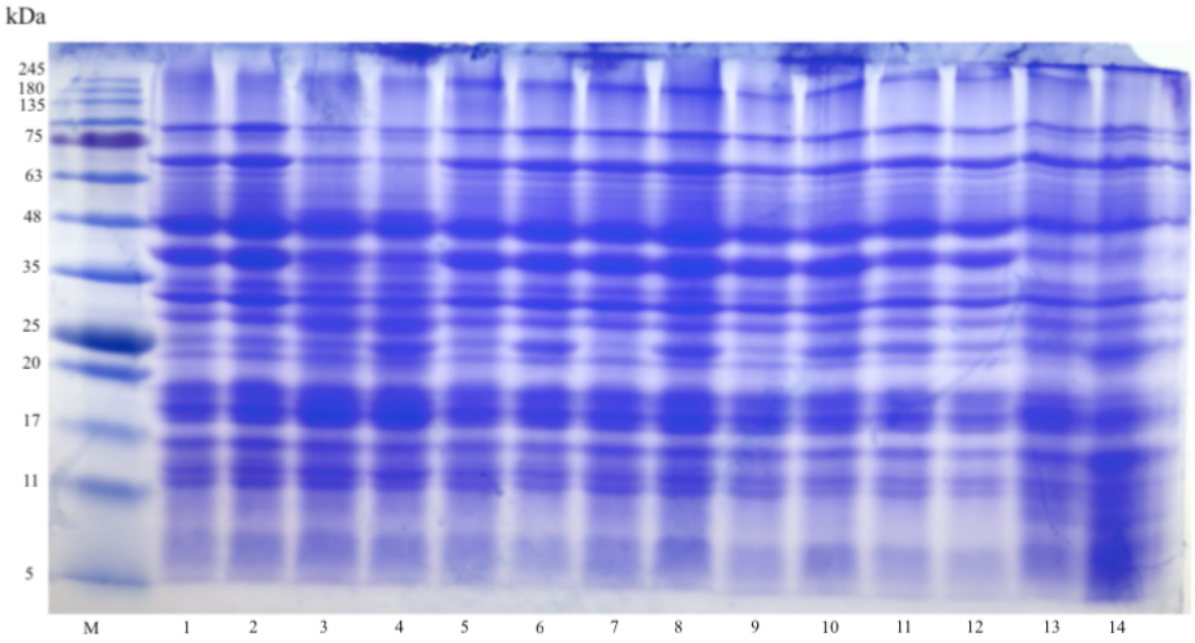

Figure 4.1.1 Representative SDS-PAGE gel of an air-classified pea protein-enriched flour enzymatically modified with different enzymes to achieve different levels of hydrolysis (DH= ~2-4% vs. 10-12%). Lanes: (M) molecular weight marker (1) unheated, pH 7.5, (2) heated, pH 7.5, (3) trypsin 2.3% DH, (4) trypsin 10%DH, (5) Savinase 2.3% DH, (6) Savinase 10.4% DH, (7) unheated, pH 6.2, (8) heated pH 6.2, (9) papain 4.0%, (10) papain 11.3 %DH, (11) unheated, pH 2.6, (12) heated, pH 2.6, (13) pepsin 2.1% DH, (14) pepsin 10.0% DH. All enzyme reactions were terminated by heating to 85°C for 30 min for trypsin, Savinase and pepsin, while papain was heated to 100°C for 30 min. Controls included: a) unheated (at pH values corresponding to those used during the enzymatic treatments, but without heating) and b) heated (heated under the same temp. and pH conditions as the enzymatic treatments, but without enzymes).

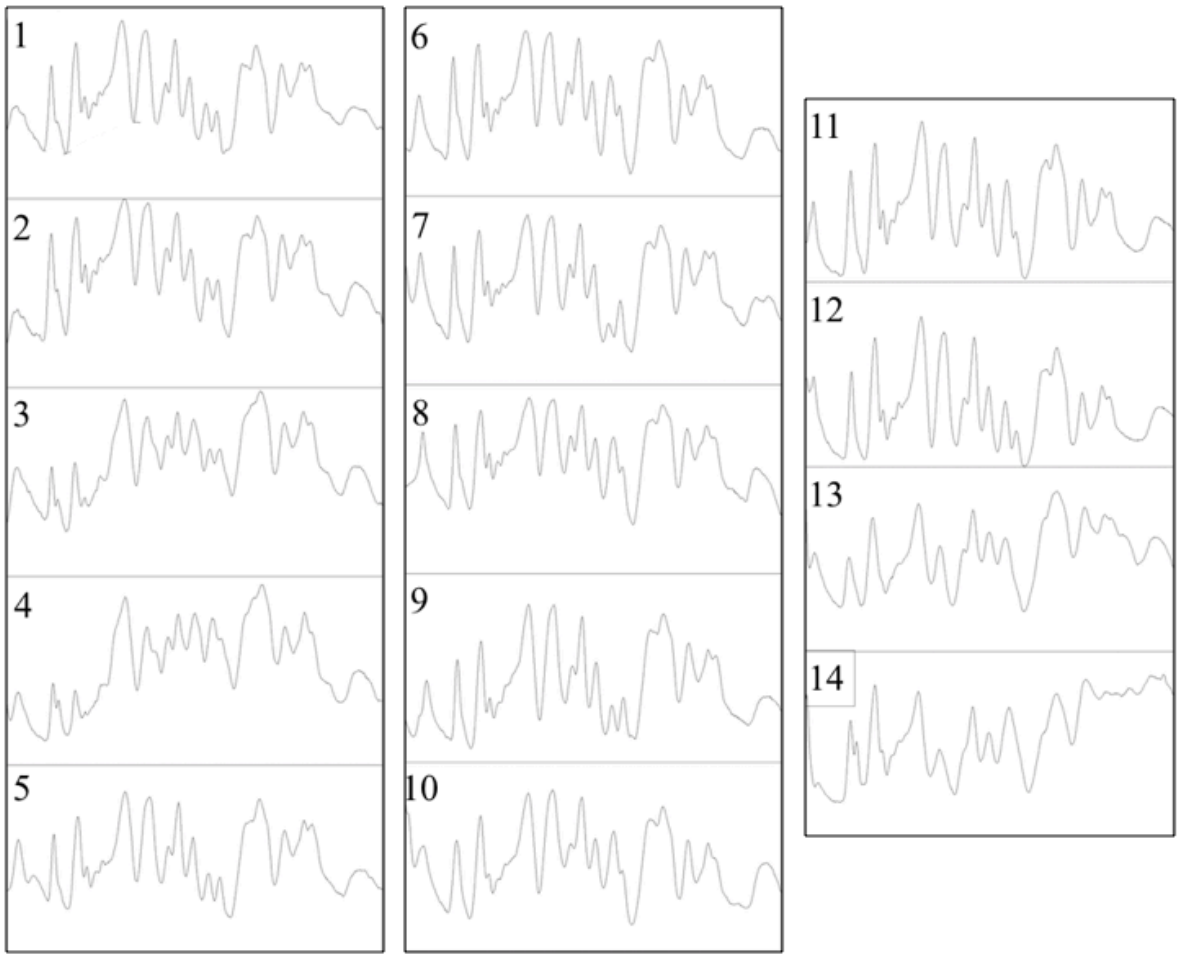

Figure 4.1.2 SDS-PAGE ImageJ chromatogram obtained gel densitometry analysis of an air-classified pea protein-enriched flour enzymatically modified with different enzymes to achieve different levels of hydrolysis (DH =~2-4% vs. 10-12%). (1) unheated, pH 7.5, (2) heated, pH 7.5, (3) trypsin 2.3% DH, (4) trypsin 10.0% DH, (5) Savinase 2.3% DH, (6) Savinase 10.4% DH, (7) unheated, pH 6.2, (8) heated pH 6.2, (9) papain 4.0% DH, (10) papain 11.3% DH, (11) unheated, pH 2.6, (12) heated, pH 2.6, (13) pepsin 2.1% DH, and (14) pepsin 10.0% DH).

Table 4.1.3 SDS-PAGE ImageJ quantification of an air-classified pea protein-enriched flour (PPEF) enzymatically modified with different enzymes to achieve different level of hydrolysis (DH = ~2-4% vs. ~10-12%).[1]

	Molecular weight (kDa) concentration (%)									
Treatment	**~93**	**~65**	**~42**	**~35**	**~31**	**~29**	**~17**	**~15**	**~8**	**~3.5**
Untreated PPEF	8.8	11.7	23.9	13.5	11.8	5.2	16.3	3.0	4.0	2.0
Trypsin (T) and Savinase (S)										
• Unheated (control)	5.8	7.6	17.1	10.2	7.5	2.8	5.4	20.2	19.6	3.9
• Heated (control)	6.9	7.8	15.3	8.9	6.7	2.6	5.6	20.7	18.9	6.5
• T (DH 2.3%)	4.2	4.3	17.8	7.3	4.9	4.1	5.2	23.9	21.4	6.5
• T (DH 10.0%)	4.5	2.5	19.8	7.1	5.2	4.4	8.4	24.2	17.6	6.5
• S (DH 2.3%)	4.1	7.1	15.5	9.8	7.2	3.3	3.9	23.8	20.8	4.5
• S (DH 10.4%)	6.3	7.8	15.7	8.9	7.1	2.7	9.0	23.1	15.0	4.4
Papain (Pa)										
• Unheated (control)	5.4	7.7	15.5	10.4	7.0	2.8	3.8	23.6	18.9	4.9
• Heated (control)	5.7	7.3	14.8	8.8	6.6	2.6	7.7	23.3	16.9	6.4
• Pa (DH 4.0%)	4.9	6.8	18.7	11.8	7.7	3.2	3.8	24.1	14.9	4.2
• Pa (DH 11.3%)	5.5	7.2	16.8	10.2	6.1	2.0	8.1	25.0	12.8	6.3
Pepsin (Pe)										
• Unheated (control)	6.5	8.2	18.4	8.5	7.5	2.9	7.0	23.3	14.5	3.1
• Heated (control)	5.2	7.3	13.2	3.0	5.9	11.6	10.3	21.8	18.3	3.4
• Pe (DH 2.1%)	4.5	6.7	12.9	2.9	4.8	12.6	10.6	21.3	20.2	3.5
• Pe (DH 10.0%)	4.4	6.6	12.2	2.8	5.5	13.7	10.8	20.7	20.6	3.6

[1]Enzyme treatments are as follows: a) trypsin and Savinase (pH 7.5, 37°C, 20 min, and 70 min); b) papain (pH 6.2, 45°C, 20 min and 40 min); and c) pepsin (pH 2.6, 37°C, 30 min and 70 min). All enzyme reactions were ceased by heating to 85°C for 30 min for trypsin, Savinase and pepsin, while papain was heated to 100°C for 30 min. Controls included: a) unheated (at pH values corresponding to the enzymatic treatments, but without heating) and b) heated (heated under the same temp. and pH conditions as the enzymatic treatments, but without enzymes). Upon completion all enzymatic treatments, the pea protein-enriched flour was pH adjusted to pH 7.0 prior to drying of the ingredient into a powder. All data is reported as the mean ± one standard deviation.

4.1.2 Physicochemical properties

The surface charge (zeta potential) and hydrophobicity of untreated and enzymatically treated PPEF is given in Table 4.1.4. The surface charge of a protein helps determined its stability in solution, where close to its isoelectric point (pI) its net neutral charge promotes protein-protein aggregation creating an unstable solution with minimum solubility). At pH values away from the pI, proteins carry a high charge leading to electrostatic repulsive forces which inhibits protein-protein aggregation and promotes protein-water interactions to allow proteins to be soluble in solution (Can Karaca *et al.*, 2011). Surface hydrophobicity also plays a significant role in governing protein-protein interactions. In general, proteins with high surface hydrophobicity tend to have reduced solubility, however can integrate well into an oil-water or air-water interface of an emulsion or foam to help lower interfacial tension to stabilize the structures. Surface hydrophobicity tends to arise mostly from the aromatic amino acids: tyrosine, tryptophan and phenylalanine. High hydrophobicity also leads to better oil holding properties of the protein. Knowledge of both properties can help understand their functional behavior in solution.

In the case of surface charge, all samples carried a negative charge at pH 7.0 (Table 4.1.4). An orthogonal individual degree of contrast analysis found that the untreated PPEF (-12.6 mV) has a significantly lower charge than the hydrolyzed PPEF (-16.3 mV; regardless of enzyme-type and degree of hydrolysis) (Table 4.1.4) ($p<0.01$). The greater charge on the hydrolyzed PPEF is due to the partial unraveling of the protein's conformation to expose buried hydrophilic amino acids, along with peptides that were cleaved off during the process. The interaction between enzyme-type and degree of hydrolysis was found not to be significant ($p>0.05$) (Table 4.1.2) therefore the main effects will be discussed differently. In the case of enzyme-type (regardless of the degree of hydrolysis), surface charge of modified PPEF was like PPEF treated with trypsin, Savinase and papain (~15.4 mV), and lower than that of PPEF treated with pepsin (~18.9 mV) ($p<0.01$) (Table 4.1.4). For the degree of hydrolysis, a slight increase in surface charge was observed from -15.7 mV to -16.6 mV as the degree of hydrolysis increased from 2-4% to 10-12% ($p<0.05$) (Table 4.1.3). Like the trend observed in this study, the zeta potential became more negative with hydrolysis for Alcalase and Flavourzyme hydrolyzed Riceberry rice bran (Thamnarathip *et al.*, 2016). However, the zeta potential of Riceberry rice bran protein was more negative than PPEF.

Table 4.1.4. The surface properties (at pH 7.0) of an air-classified pea protein-enriched flour (PPEF) enzymatically modified with different enzymes to achieve different level of hydrolysis (DH = ~2-4% vs. ~10-12%).

Treatment	Zeta Potential (mV)	Surface hydrophobicity (A.U. arbitrary units)
Untreated PPEF	-12.6 ± 0.2	13.3 ± 0.4
Trypsin (T) and Savinase (S)		
• Unheated (control)	-13.9 ± 0.3	24.5 ± 2.0
• Heated (control)	-14.3 ± 0.1	21.6 ± 1.5
• T (DH 2.3)	-15.5 ± 0.3	22.8 ± 0.9
• T (DH 10.0%)	-15.6 ± 0.2	40.2 ± 4.7
• S (DH 2.3%)	-14.5 ± 0.5	26.8 ±1.2
• S (DH 10.4%)	-16.8 ± 0.1	44.5 ± 0.9
Papain (Pa)		
• Unheated (control)	-13.9 ± 0.1	23.2 ± 1.2
• Heated (control)	-13.9 ± 0.8	35.9 ± 1.8
• Pa (DH 4.0%)	-14.0 ± 0.3	35.7 ± 0.9
• Pa (DH 11.3%)	-15.9 ± 0.3	48.5 ± 1.1
Pepsin (Pe)		
• Unheated (control)	-14.3 ± 0.8	25.0 ± 1.0
• Heated (control)	-14.1 ± 0.8	41.6 ± 0.3
• Pe (DH 2.1%)	-19.0 ± 0.3	46.5 ± 0.1
• Pe (DH 10.0%)	-18.9 ± 0.6	46.6 ± 0.3

[1]Enzyme treatments are as follows: a) trypsin and Savinase (pH 7.5, 37°C, 20 min, and 70 min); b) papain (pH 6.2, 45°C, 20 min and 40 min); and c) pepsin (pH 2.6, 37°C, 30 min and 70 min). All enzyme reactions were ceased by heating to 85°C for 30 min for trypsin, Savinase and pepsin, while papain was heated to 100°C for 30 min. Controls included: a) unheated (at pH values corresponding to the enzymatic treatments, but without heating) and b) heated (heated under the same temp. and pH conditions as the enzymatic treatments, but without enzymes). Upon completion all enzymatic treatments, the pea protein-enriched flour was pH adjusted to pH 7.0 prior to drying of the ingredient into a powder. All data is reported as the mean ± one standard deviation.

The untreated sample had a zeta potential of ~-30 mV and decreased to >-40 mV when hydrolyzed compared to the ~-13 mV for untreated and -18 mV for enzyme treated PPEF. Similar values to this were observed for lentil protein isolate by Avramenko *et al.* (2013). The authors found a decrease in the zeta potential of lentil protein isolate when treated with trypsin up to 10% DH. Untreated samples were ~-35 mV and decreased to -38 mV with enzyme treatment. The difference

between the samples could have resulted from the different substrate and concentration of carbohydrates in the isolates (>80% protein) that were present in the PPEF (~50% protein). The effect of decreasing zeta potential could have resulted from the increase in exposure of charged amino acids that may have been buried within the protein structure before hydrolysis.

A flaxseed protein concentrate with a similar protein content (51%) to that of the PPEF used in this study (~52%) had a zeta potential of -11 mV in a study by Tirgar *et al.* (2017). Pea protein concentrate was used as a control (76% protein), and the zeta potential was determined to be -16 mV, which is comparable to that found in this study of PPEF. These results suggest that the protein ingredient (*i.e.*, flour, concentrate or isolate) influence the zeta potential. The surface charge of PPEF was found to be lower than that found in literature for pea protein (-23 to -30 mV), Lam *et al.* (2016) of -44 to -47 mV for other pulse proteins such as chickpea or faba bean, respectively. When the surface charge of protein is relatively low, the proteins can aggregate or interact with each other from lack of repulsion between them. Coupled with increased hydrophobicity, the proteins can interact through hydrophobic interactions and lead to destabilization and lack of solubility. These intrinsic properties of the protein may have originated from the initial processing of air-classified pea protein concentrate.

The enzymes used to hydrolyze PPEF altered the protein structure specific to their substrate preferences, which varied between the enzymes. The enzymes used in this study to modify PPEF are endopeptidases which hydrolyze proteins at away from the terminal amino acids. Trypsin, Savinase and papain followed the trend where the higher hydrolyzed protein had a higher surface hydrophobicity. Enzymes are specific to the area in which they cleave, therefore the resulting PPEF can have unique surface properties. Trypsin and Savinase are serine proteases; while trypsin prefers peptide bonds next to lysine and arginine and hydrophobic residues, Savinase can cleave at random respectively. Papain is a thiol (cysteine) protease where cysteine is in its active center, and it has differences in the optimum pH and temperature, leading different hydrolysis pattern from the serine proteinases. Pepsin is an aspartic protease, which works better under acidic conditions and has less site preferences among peptide bonds.

In the case of surface hydrophobicity, an orthogonal individual degree of contrast analysis found that the untreated PPEF (13.3 A.U.) has a significantly lower charge than the hydrolyzed PPEF (~38.9 A.U.); regardless of enzyme-type and degree of hydrolysis ($p<0.001$) (Table 4.1.4). During hydrolysis, a greater amount of previously buried hydrophobic amino acids are thought to

be more exposed. The interaction between enzyme type and degree of hydrolysis was found to be significant ($p<0.001$) (Table 4.1.2). For all enzymes except for pepsin, hydrophobicity was found to increase as the degree of hydrolysis increased from 2-4% to 10-12%. For instance, PPEF increased from 22.8 to 40.2 A.U., from 26.8 to 44.5 A.U., and from 35.7 to 48.5 A.U., when treated with trypsin, Savinase and papain, respectively (Table 4.1.4). In contrast, the hydrophobicity of pepsin-treated PPEF was independent of the degree of hydrolysis (46 A.U.) (Table 4.1.4). A similar increase in surface hydrophobicity was found in a study by Wu *et al.* (1998), where hydrolyzed soy protein isolate (SPI) modified by papain had a higher surface hydrophobicity compared to the untreated SPI resulting from the exposure of hydrophobic groups. Increasing the DH of the protein to 10-12%, increased the surface hydrophobicity, suggesting that further hydrolysis unravels the protein structure allowing for more hydrophobic groups to be exposed.

In the case of pepsin-treated PPEF, the hydrophobicity did not increase with further hydrolysis and remained at ~46 A.U. However, some researchers have found that more extensively hydrolyzed protein has been unable to fully measure the hydrophobic patches on the protein due to the smaller peptide size, or interaction between hydrophobic residues, where ANS is unable to measure (Wu *et al.*, 1998). Pepsin-treated PPEF gave distinctly different surface properties than the effects of the other three enzymes in terms of both the charge and the hydrophobicity. For instance, it gave the highest surface charge of -18.9 mV, much higher than the other three (-15.4 mV), and it also showed a high hydrophobicity (46 A.U.) with low degree of hydrolysis (2-4%) whereas the others low. Differences between papain and pepsin could potentially be explained by their mode of action. Pepsin only can hydrolyze pea proteins under acidic pH (pH 2.6), where both the enzyme and the low pH can induce changes to the protein conformation and ionizable groups on the protein. Papain is active at near neutral pH (pH 6.2), however is more heat-stable than the other enzymes requiring temperatures to be raised to 100°C vs. 85°C to inactive the enzyme. Both low pH and higher temperatures can impact protein unfolding, which can lead to differences in protein unfolding. Differences in enzyme specificity can also lead to different spatial areas of the protein being hydrolyzed, exposing other hydrophobic and hydrophilic amino acids despite having similar overall degree of hydrolysis. The specificity of trypsin, Savinase and pepsin tends to be greater than papain, which cleaves proteins in a random fashion.

4.1.3 Functionality

(a) Solubility

The solubility of untreated and enzymatically treated PPEF is given in Table 4.1.5. Solubility of a protein is an important precursor to other functional properties, such as foaming and emulsification. Solubility is defined as the percentage of protein capable of being dissolved within an aqueous solution (Hall, 1996). Higher solubility typically occurs away from the pI of the protein where electrostatic repulsive forces are greatest, and protein-solvent interactions is favored over protein-protein interactions (Ladjal-Ettoumi *et al.*, 2016). An individual degree of freedom orthogonal contrast analysis found that solubility of the untreated PPEF were significantly higher than the hydrolyzed samples (regardless of the enzyme-type and degree of hydrolysis) at each pH ($p<0.001$) (Table 4.1.2, 4.1.5). For instance, untreated PPEF at pH 4.0, 7.0 and 10.0 were 56.8%, 79.9% and 93.6%, respectively, whereas all hydrolyzed samples were lower at corresponding pH values with solubilites of 36.0%, 44.8% and 71.4%, respectively. Most reported hydrolysates have shown an increase in solubility compared to the untreated samples, and show less of a U-shaped solubility profile, and even solubility across all pH values (Jung *et al.*, 2005; Yin *et al.,* 2008; Zhao *et al.*, 2010). However, in the present study, the solubility decreased with enzyme treatment. A decrease in the solubility of pea and broad bean protein was observed by Tsoukala *et al.* (2006) when modification of the legume protein was induced through autolysis. Autolyzed pea protein solubility decreased from 86% to 50%, and broad bean decreased from 65% to 25% at pH 6.5. The decrease in solubility was thought to occur because of increased hydrophobicity. The lower solubility with the hydrolyzed PPEF is hypothesized caused by the liberation of smaller peptides from the protein chain, and/or the exposure of hydrophobic amino groups after partially unfolding, which then aggregated via protein-protein interactions to fall out of solution. In the present study, surface charge of the untreated and hydrolyzed PPEF remains relatively low (~ -12 to -20 mV) at pH 7.0, whereas the surface hydrophobicity increases substantially upon hydrolysis (Table 4.1.4), suggesting that aggregation driven by hydrophobic interactions is the most likely mode of action leading to the reduced solubility.

The orthogonal analysis also found the degree of hydrolysis ($p<0.05$) to be significant, along with interaction between enzyme-type and pH to be significant ($p<0.05$) therefore their individual effects will not be discussed separately (Table 4.1.2).

Table 4.1.5. Protein solubility (%) of an air-classified pea protein-enriched flour (PPEF) enzymatically modified with different enzymes to achieve different degree of hydrolysis at pH 4.0, 7.0 and 10.0.

Treatment[1]	Solubility (%)		
	pH 4.0	**pH 7.0**	**pH 10.0**
Untreated PPEF	56.8 ± 2.2	79.9 ± 2.9	93.6 ± 2.9
Trypsin (T) and Savinase (S)			
• Unheated (control)	46.4 ± 0.6	58.1 ± 1.6	89.7 ± 1.8
• Heated (control)	35.6 ± 1.0	43.1 ± 0.6	78.0 ± 1.1
• T (DH 2.34)	42.5 ± 0.8	49.0 ± 1.4	78.5 ± 1.1
• T (DH 10.02%)	37.3 ± 0.2	47.7 ± 1.2	80.0 ± 0.9
• S (DH 2.31%)	31.2 ± 0.6	35.0 ± 1.6	61.9 ± 0.4
• S (DH 10.39%)	34.4 ± 0.5	45.5 ± 1.6	69.3 ± 0.1
Papain (Pa)			
• Unheated (control)	37.6 ± 1.1	48.7 ± 0.8	89.8 ± 1.0
• Heated (control)	35.5 ± 0.7	45.6 ± 1.9	76.4 ± 1.6
• Pa (DH 3.96%)	36.5 ± 0.9	44.3 ± 0.4	59.6 ± 1.1
• Pa (DH 11.34%)	36.3 ± 1.2	44.5 ± 1.2	62.6 ± 0.9
Pepsin (Pe)			
• Unheated (control)	35.0 ± 0.7	48.3 ± 0.7	88.8 ± 1.0
• Heated (control)	30.7 ± 0.1	45.6 ± 1.9	75.6 ± 0.9
• Pe (DH 2.12%)	34.1 ± 0.5	41.6 ± 0.4	67.0 ± 1.2
• Pe (DH 10.03%)	36.0 ± 1.6	50.4 ± 0.1	92.3 ± 1.2

[1]Enzyme treatments are as follows: a) trypsin and Savinase (pH 7.5, 37°C, 20 min, and 70 min); b) papain (pH 6.2, 45°C, 20 min and 40 min); and c) pepsin (pH 2.6, 37°C, 30 min and 70 min). All enzyme reactions were ceased by heating to 85°C for 30 min for trypsin, Savinase and pepsin, while papain was heated to 100°C for 30 min. Controls included: a) unheated (at pH values corresponding to the enzymatic treatments, but without heating) and b) heated (heated under the same temp. and pH conditions as the enzymatic treatments, but without enzymes). Upon completion all enzymatic treatments, the pea protein-enriched flour was pH adjusted to pH 7.0 prior to drying of the ingredient into a powder. All data is reported as the mean ± one standard deviation.

In terms of the degree of hydrolysis of PPEF (regardless of the enzyme-type or pH), solubility was found to increase slightly from 50.1% to 53.0% as the degree of hydrolysis increased from 2-4% to 10-12%, respectively (Table 4.1.5). The increased solubility resulting from a higher extent of hydrolysis maybe due to a presumed reduction in molecular mass, as well as an increased amount of exposed hydrophilic amino acids at the surface to favor more protein-solvent interactions hydrolysis of PPEF (regardless of the enzyme-type or pH). The increased solubility resulting from a higher extent of hydrolysis maybe due to a presumed reduction in molecular mass, as well as an increased amount of exposed hydrophilic amino acids at the surface to favor more protein-solvent interactions.

Overall, solubility was lowest at pH 4.0 which is close to the isoelectric point of pea protein (pH 4.6; Barac *et al.*, 2015), and then increased at pH 7.0 and then again at pH 10.0 as the solution pH moved further away from the pI. As this happens the protein assumes more of a negative charge and protein-solvent interactions are favored over protein-protein interactions leading to the increased solubility. Although each hydrolyzed PPEF sample followed the same trend, the magnitude of change was quite different. For instance, trypsin- and pepsin-treatment showed the greatest magnitude of change, increasing from 39.9% to 79.3%, and 35.0% to 79.6% soluble protein, respectively as pH increased from 4.0 to 10.0. Savinase- and papain-treatment had a smaller rise in solubility, increasing from 32.8% to 65.6% and 36.4% to 61.1% soluble protein, respectively. Differences in modes of action of the enzymes could alter the solubility of the proteins each enzyme cleaves at specific locations to alter its conformation to give different surface properties that could impact protein-protein and protein-solvent interactions.

The untreated PPEF had the highest solubility at all pH values, and declined with modification resulting from the increased exposure of hydrophobicity. The higher range of hydrolysis (10-12%) favored the release of soluble peptides from the insoluble aggregate/precipitate that resulted from the increased solubility after limited hydrolysis (2-4%). Ventureira *et al.* (2010) found a decrease in solubility of amaranth protein isolate at pH 2.0, 6.3, 8.0 after limited Alcalase treatment (1.7% DH), and then with further hydrolysis (9.6% DH) the solubility increased. The decrease in solubility from the untreated sample resulted from the enzymes proteolytic activity which increased hydrophobicity, which favored protein-protein aggregation through hydrophobic interactions, leading to precipitation of protein out of solution. However, with higher degree of hydrolysis, the proteolytic action of the enzyme used could have

increased the exposure of ionizable and carboxyl groups that favor interactions in solution (Panyam & Kilara, 1996). Further hydrolysis from 3-5% to 7-10% DH increased the solubility of papain-hydrolyzed kidney bean protein isolates, where the increase in solubility had resulted from the unfolding of the protein to exposure of polar amino acids where they can interact with the water via hydrogen bonds and electrostatic interactions (Wani *et al.*, 2015). Similarly, sunflower protein isolate solubility increased with higher extent hydrolysis with Alcalase, and Flavourzyme hydrolysis. The increase in hydrolysis of sunflower protein samples for both enzymes increased the solubility as a result of the release of soluble peptide from the protein structure (Villanueva *et al.*, 1999). Therefore, this suggests that when proteins are more extensively hydrolyzed, the solubility might increase, and potentially could have better resulting functional properties. Therefore, even though the solubility of PPEF decreased compared to the untreated sample, further hydrolysis could increase the solubility, which was shown in all pH values tested with the DH at 10-12%. This suggests that the further the PPEF is hydrolyzed to break apart the aggregate that formed after limited hydrolysis, the better the resulting solubility will be. It was proposed by Guan *et al.* (2007) that the improvements with increasing hydrolysis could be attributed to the unravelling of the compact protein structure, decrease in size of peptide chain and exposure of charged/polar groups.

Another explanation for the reduced solubility is heat denaturation. Heat-induced denaturation is a common factor leading to decreased protein solubility. High temperatures were used to terminate the enzymatic reaction (85°C for Savinase, trypsin and pepsin and 100°C for papain for 30 min) and therefore could have resulted in denaturation to the protein. When heated, proteins can unfold and exposure hydrophobic portions of their structure, and continued heat processing can lead to permanent changes in the structure. When hydrophobic residues are exposed they can lead to an aggregate formation and precipitation out of solution. Therefore, as hydrolysis exposed hydrophobic regions on the protein, and then was heated after hydrolysis to denature the enzyme, the PPEF may have denatured as well and aggregated and precipitated out of solution.

(b) Oil holding capacity and water holding capacity

The water holding (WHC) and oil holding (OHC) capacities of untreated and enzymatically treated PPEF is given in Table 4.1.6. These parameters relate to the amount of water or oil (in grams) a given amount of protein can hold or entrap within its matrix (Owusu-Ansah *et al.*, 1991).

For WHC, an orthogonal individual degree of freedom contrast analysis found that the untreated PPEF (0.60 g/g) lower than that of the hydrolyzed samples (1.69 g/g) ($p<0.001$) (Table 4.1.6 and 4.1.2). The analysis also found the effect of enzyme-type ($p<0.001$) and degree of hydrolysis ($p<0.001$) also had significant effects on WHC, but not their interaction ($p>0.05$). Trypsin-, papain-, pepsin- and Savinase-treated PPEF (regardless of the degree of hydrolysis) had WHC values of 1.55, 1.81, 1.92, and 1.50 g/g, respectively. Pepsin- and papain-treated PPEF showed much higher WHC than the other two enzyme treatment, possibly because upon partial unfolding a greater amount of ionizable groups were exposed or micro-capillaries developed with the change in conformation to enable higher amounts of water to bind.

Trypsin- and Savinase-treatment, which showed much lower WHC, have the same mode of action (serine protease), thus similar reaction conditions, suggesting that the two enzymes may not create as many ionizable groups or microcapillaries upon partial hydrolysis at their sites of attack. The average WHC also was found to increase from ~1.63 g/g to ~1.79 g/g as the degree of hydrolysis increased from 2-4% to 10-12% presumed caused by the greater amount of partial unfolding of the protein (Table 4.1.6).

For OHC, an orthogonal individual degree of freedom contrast analysis found that the untreated PPEF (0.74 g/g) was lower to that of the hydrolyzed samples (1.08 g/g; $p<0.001$) (Table 4.1.6 and 4.1.2). The analysis also showed that only the degree of hydrolysis of the protein impacted OHC ($p<0.001$), and not the enzyme-type or the interaction with the degree of hydrolysis ($p>0.05$) (Table 4.1.5 and 4.1.2). OHC was found to increase from ~0.97 g/g for the PPEF with 2-4% degree of hydrolysis to ~1.20 g/g for the 10-12% (Table 4.1.5). The increase in OHC for further hydrolyzed samples is thought to be associated with the large increased in surface hydrophobicity between untreated and hydrolyzed samples (13.3 vs 38.9 A.U.), and those with increased levels of hydrolysis (32.9 vs 44.9 A.U.) (Table 4.1.4). Like WHC, the unraveling of the protein conformation would also produce micro-capillaries that could entrap increased levels of oil.

Stone *et al.* (2015) found similar OHC and WHC of pea protein isolate, (1.07 to 1.40 g/g and 1.91 to 2.37 g/g, respectively) to the hydrolyzed pea protein-enriched flour. The OHC and WHC increased for all hydrolyzed samples, and increased with further DH. The modification to the protein resulting from hydrolysis changes the surface properties of the protein exposing polar, and non-polar amino acids, enabling it to entrap oil or water within its structure..

Table 4.1.6. Water and oil holding capacity of an air-classified pea protein-enriched flour (PPEF) enzymatically modified with different enzymes to achieve different level of hydrolysis.

Treatment[1]	Water hydration capacity (g/g)	Oil holding capacity (g/g)
Untreated PPEF	0.60 ± 0.01	0.74 ± 0.03
Trypsin (T) and Savinase (S)		
• Unheated (control)	1.27 ± 0.02	0.98 ± 0.07
• Heated (control)	1.29 ± 0.03	1.06 ± 0.03
• T (DH 2.34)	1.49 ± 0.01	0.95 ± 0.02
• T (DH 10.02%)	1.60 ± 0.06	1.18 ± 0.08
• S (DH 2.31%)	1.38 ± 0.05	1.06 ± 0.03
• S (DH 10.39%)	1.61 ± 0.02	1.10 ± 0.03
Papain (Pa)		
• Unheated (control)	1.34 ± 0.06	0.92 ± 0.03
• Heated (control)	1.68 ± 0.06	0.87 ± 0.13
• Pa (DH 3.96%)	1.59 ± 0.07	0.89 ± 0.09
• Pa (DH 11.34%)	2.04 ± 0.07	1.46 ± 0.03
Pepsin (Pe)		
• Unheated (control)	1.38 ± 0.08	0.99 ± 0.06
• Heated (control)	1.60 ± 0.03	0.89 ± 0.08
• Pe (DH 2.12%)	1.89 ± 0.03	0.97 ± 0.08
• Pe (DH 10.03%)	1.94 ± 0.08	1.05 ± 0.09

[1]Enzyme treatments are as follows: a) trypsin and Savinase (pH 7.5, 37°C, 20 min, and 70 min); b) papain (pH 6.2, 45°C, 20 min and 40 min); and c) pepsin (pH 2.6, 37°C, 30 min and 70 min). All enzyme reactions were ceased by heating to 85°C for 30 min for trypsin, Savinase and pepsin, while papain was heated to 100°C for 30 min. Controls included: a) unheated (at pH values corresponding to the enzymatic treatments, but without heating) and b) heated (heated under the same temp. and pH conditions as the enzymatic treatments, but without enzymes). Upon completion all enzymatic treatments, the pea protein-enriched flour was pH adjusted to pH 7.0 prior to drying of the ingredient into a powder. All data is reported as the mean ± one standard deviation.

Deng *et al.* (2016) determined that the use of a protease from *Aspergillus usamii* on wheat gluten improved the WHC from 1.47 to 1.75 g/g and improved the OHC from 0.92 to 2.91 g/g. The authors suggested that the significant improvements in OHC were a result from the suggested increase in hydrophobicity from enzymatic treatment. Peragio *et al.* (1997) also attributed higher OHC of hydrolyzed pea flour compared to untreated flour, to increases in hydrophobic amino acids on the peptide chain resulting from the action of protease from *Aspergillus saitoi* Similarly, the increase in hydrophobicity resulting from the exposure of buried hydrophobic amino acids was thought to be responsible for the increase in OHC for hydrolyzed potato protein concentrate modified with Alcalase in a study by Miedzianka *et al.* (2014). The initial WHC observed in the untreated sample could make the food product more sensitive to storage humidity, and while a high WHC (<4.0) was not observed, it could decrease the moisture content of other ingredients in the food, altering the texture and mouthfeel (Zayas, 1997). In both cases of OHC and WHC, papain treatment of PPEF had the highest increase at 10-12% DH. The improvements of papain treatment could be explained by the non-specific proteolytic action, where papain cleaves at random, opening the structure of the protein.

Wani *et al.* (2015), papain-treated kidney bean proteins hydrolyzed at [1/1000] increased the OHC and WHC properties. However, a higher OHC and WHC (<4.0-5.0 g/g) was observed, which may be dependent on the type of protein used as a substrate. The authors also investigated how the proteolysis time would affect the OHC and WHC. Hydrolysis time intervals of 0, 30, and 60 min were investigated and the investigators found that both OHC and WHC increased with increasing time (higher percent DH). Vioque *et al.* (2000) found significant increases in both OHC and WHC in rapeseed protein hydrolyzed with both Alcalase and protease from *Bacillus licheniformis.* The OHC of untreated rapeseed was comparable to that of the untreated PPEF used in this study (0.63 g/g), and increased at 3.1% DH to 1.55 g/g. The WHC of hydrolyzed rapeseed was much higher for both the untreated sample (1.31 g/g) and increased to 5.85 g/g at 3.1% DH. Similar to the OHC of PPEF, the increase in degree of hydrolysis increased both the OHC and WHC.

Oat bran concentrate was hydrolyzed with trypsin in a study by Guan *et al.* (2007) and found that the WHC increased with percent DH, where the untreated sample was 1.94 g/g and increased to 2.13 g/g at 4.1% DH, 2.27 g/g at 6.4% DH and 2.25 g/g at 8.3% DH. The authors thought that the WHC may have increased from the heat treatment applied to deactivate the enzyme. The heat

step in the presence of water could result in starch swelling leading to gelatinization and further increased with enzyme treatment and degree of hydrolysis. This could potentially be occurring in the PPEF used in the study, as the protein contents are only ~50%, therefore ~40% could be carbohydrates which could contribute to starch swelling and increasing the WHC.

(c) Emulsifying properties

The emulsifying activity (EAI) and stability (ESI) indices for untreated and hydrolyzed PPEF as a function of pH is given in Table 4.1.7. EAI is the interfacial area that the protein can stabilize when forming an emulsion and is measured based on the turbidity of the solution (Pearce & Kinsella, 1978; Stone *et al.*, 2015). ESI is the resistance of a stable emulsion to separate into two phases; oil and water occurring through creaming, flocculation and coalescence, over a set time (10 min) (Pearce & Kinsella, 1978). Orthogonal contrast analysis found EAI for the hydrolyzed PPEF was lower at each pH than the untreated sample, where at pH 4.0 EAI was reduced from 33.1 to 29.4 m^2/g with hydrolysis, at pH 7.0 from 82.0 to 52.5 m^2/g, and at pH 10.0 from 99.5 to 88.7 m^2/g ($p<0.01$) (Table 4.1.7). The reduced EAI values for the hydrolyzed samples are hypothesized to be associated with the reduced solubility of the proteins, where they would take longer to diffuse to the oil-water interface than a more soluble protein. The analysis also revealed enzyme-type and the interaction between enzyme and pH to significantly affect EAI ($p<0.001$) (Table 4.1.2). For all enzyme-treated PPEF, EAI increased as the pH moved away from the pI of the protein, however the magnitude of changes was enzyme-dependent. Improved emulsion forming properties at higher pH values is thought to be associated with improved solubility of the protein to enable faster rates of diffusion to the interface. Trypsin-treated PPEF had EAI values of 37.4, 80.5 and 115.4 m^2/g at pH 4.0, 7.0 and 10.0, respectively. Savinase-treated PPEF showed a similar trend, except at pH 7.0 where the magnitude was lower. EAI values were 36.5, 46.4 and 115.7 m^2/g at pH 4.0, 7.0 and 10.0, respectively. In contrast, papain and pepsin-treated PPEF had EAI values much lower. EAI values for papain-treated PPEF were 23.1, 41.0, and 51.0 m^2/g at pH 4.0, 7.0 and 10.0, respectively, whereas EAI for papain-treated PPEF were 20.7, 42.1 and 72.7m^2/g, respectively (Table 4.1.7). As previously discussed, trypsin and Savinase are both serine proteases , with similar reactions conditions which have a different mode of action than both papain and pepsin, which would result in different spatial areas on the protein where unfolding occurred.

Table 4.1.7. Emulsion activity index (m^2/g) and emulsion stability index (min) an air-classified pea protein-enriched flour (PPEF) enzymatically modified[1] with different enzymes to achieve different level of hydrolysis at pH 4.0, 7.0 and 10.0.

Treatment	Emulsion activity index (m^2/g)			Emulsion stability index (min)		
	pH 4.0	pH 7.0	pH 10.0	pH 4.0	pH 7.0	pH 10.0
Untreated PPEF	33.2 ± 0.9	82.0 ± 2.7	99.5 ± 0.9	23.1 ± 0.9	55.5 ± 2.1	46.8 ± 0.5
Trypsin (T) and Savinase (S)						
• Unheated (control)	141.1 ± 1.3	110.0 ± 4.2	108.1 ± 3.7	10.3 ± 0.8	40.3 ± 1.3	19.8 ± 0.7
• Heated (control)	40.9 ± 1.4	107.0 ± 2.2	105.4 ± 2.2	11.3 ± 0.9	36.2 ± 0.5	19.6 ± 0.5
• T (DH 2.3%)	40.3 ± 1.9	105.4 ±1.3	121.0 ± 1.3	14.4 ± 1.0	30.3 ± 0.2	19.0 ± 1.1
• T (DH 10.0%)	34.6 ± 0.4	55.7 ± 1.7	109.8 ± 3.0	15.9 ± 0.8	32.4 ± 1.2	32.7 ± 1.6
• S (DH 2.3%)	30.0 ± 1.9	45.9 ± 1.6	114.1 ± 0.7	12.0 ± 0.7	29.3 ± 1.1	29.0 ± 1.0
• S (DH 10.4%)	43.0 ± 0.7	47.0 ± 1.3	117.3 ± 2.8	14.0 ± 0.3	28.2 ± 0.7	35.5 ± 1.4
Papain (Pa)						
• Unheated (control)	42.6 ± 1.7	78.0 ± 0.4	106.2 ± 2.4	24.0 ± 1.0	33.6 ± 1.1	30.2 ± 0.6
• Heated (control)	40.9 ± 0.7	48.8 ± 1.7	62.2 ± 1.0	17.1 ± 1.1	36.8 ± 0.5	43.2 ± 1.0
• Pa (DH 4.0%)	21.3 ± 0.6	35.5 ± 1.0	45.1 ± 1.7	25.4 ± 0.8	33.2 ± 0.8	44.0 ± 0.5
• Pa (DH 11.3%)	24.8 ± 1.0	46.5 ± 1.6	57.0 ± 2.5	26.1 ± 1.3	31.7 ± 0.9	42.9 ± 0.4
Pepsin (Pe)						
• Unheated (control)	22.7 ± 0.7	42.6 ± 1.7	79.5 ± 0.6	10.0 ± 0.56	24.0 ± 0.3	12.5 ± 1.0
• Heated (control)	24.6 ± 1.6	47.0 ± 1.9	70.9 ± 1.6	13.0 ± 1.01	11.1 ± 1.4	8.1 ± 0.2
• Pe (DH 2.1%)	22.3 ± 1.3	43.2 ± 1.7	75.5 ± 1.0	7.4 ± 0.82	7.6 ± 0.8	11.9 ± 1.0
• Pe (DH 10.0%)	19.0 ± 1.0	41.1 ± 1.3	69.9 ± 0.4	18.9 ± 1.14	30.2 ± 0.6	20.4 ± 1.7

[1]Enzyme treatments are as follows: a) trypsin and Savinase (pH 7.5, 37°C, 20 min, and 70 min); b) papain (pH 6.2, 45°C, 20 min and 40 min); and c) pepsin (pH 2.6, 37°C, 30 min and 70 min). All enzyme reactions were ceased by heating to 85°C for 30 min for trypsin, Savinase and pepsin, while papain was heated to 100°C for 30 min. Controls included: a) unheated (at pH values corresponding to the enzymatic treatments, but without heating) and b) heated (heated under the same temp. and pH conditions as the enzymatic treatments, but without enzymes). Upon completion all enzymatic treatments, the pea protein-enriched flour was pH adjusted to pH 7.0 prior to drying of the ingredient into a powder. All data is reported as the mean ± one standard deviation.

For ESI, the orthogonal contrast found ESI for the hydrolyzed PPEF was lower at each pH than the untreated sample, where at pH 4.0 ESI was reduced from 23.1 to 16.8 min with hydrolysis, at pH 7.0 from 55.5 to 27.9 min, and at pH 10.0 from 46.8 to 29.4 min ($p<0.001$) (Table 4.1.7). The reduction in emulsion stability indices with the hydrolysis samples maybe due to the presence of peptides that were cleaved off that would rapidly diffuse to the interface, and then later displaced by the larger slower diffusing proteins. Although the surface hydrophobicity on the proteins are greater it is believed based on the solubility data, that larger aggregates are re-forming in solution which may lead to poorer integration into the interface than untreated proteins. The analysis also showed that for the hydrolyzed PPEF, only the degree of hydrolysis and pH significantly affected ESI ($p<0.001$) (Table 4.1.2). For instance, ESI was determined to be 16.8, 27.9 and 29.4 min at pH 4.0, 7.0 and 10.0 respectively irrespective of the enzyme-type used and the degree of hydrolysis ($p<0.001$) (Table 4.1.7). Furthermore, ESI was found to increase as the degree of hydrolysis increased from 2-4% (22.0 min) to 10-12% (27.4 min) ($p<0.01$) (Table 4.1.7). Stability also improved for the emulsions at pH values away from the pI where electrostatic repulsive forces dominate.

The decreases in EAI could be a result of the exposure of hydrophilic moieties from enzymatic hydrolysis. This change in protein structure, where more polar, ionizable and carboxyl groups exposure would favor interaction with the aqueous phase. Bentacur-Acona *et al.* (2009) suggested that the decrease in EAI in the hydrolyzed *Phaseolus lunatus* protein, hydrolyzed by Flavourzyme and Alcalase compared to the untreated isolate sample. The authors attributed the decrease to the increase in hydrophilic moieties. Additionally, the authors found a similar decrease in ESI with increasing hydrolysis, like that of trypsin, and pepsin hydrolyzed PPEF in this study. Comparing the two different extents of hydrolysis for each enzyme suggested that the higher degree of hydrolysis (>10%) could facilitate faster diffusion of peptides to the interface, in addition to the increased hydrophilic moieties. Bentacur-Acona *et al.* (2009) similarly found that the ESI of hydrolyzed *Phaseolus lunatus* had lower stability at higher DH, which was attributed to the decrease in molecular weight of the protein, which is considered an important determinant of emulsion stability. Similarly, Zhao *et al.* (2011) found a significant decrease in EAI of peanut protein isolate hydrolyzed with Alcalase. EAI at pH 5.0 decreased from 30.7 m^2 /g (untreated) to 11.0 m^2 /g after 2.1% DH, and then increased to 16.3 m^2 /g after 3.6% DH. Similar trends were observed at pH 7.0, where EAI decreased from 56.2 m^2 /g to 39.2 m^2 /g after 2.1% DH, and then

decreased to 29.3 m^2 /g after 3.6% DH. However, at 5.4% DH the differences were not significant from the 3.6% DH sample at both pH values tested. Compared to the results in this study, there was an initial decline in EAI after 2-4% DH and then an increased after 10-12% DH for papain-, and Savinase-treated samples. However, a decrease in ESI was observed for 2-4%DH Savinase- and papain-treated samples compared to the untreated sample, and further decreased after 10-12% DH. Avramenko *et al.* (2013) found that trypsin hydrolysis on lentil protein isolate decreased the EAI and ESI. EAI decreased from ~51 to ~47 m^2/g, and ESI decreased from ~12 to 11 min at pH 7.8. When the emulsifying properties improved with a higher percent DH, it is thought to result as a reduction in molecular weight, allowing better alignment at the oil-water interface. However, a higher percent DH reduced the emulsifying properties due to more hydrophilic peptides being liberated from the protein where they are unable to create the viscoelastic film needed to prevent coalescence of oil droplets (Avramenko *et al.* 2013). However, as there are difference trends occurring based on the enzyme choice, the changes and differences at 2-4% and 10-12% are a result of the mode of action of the enzyme used and the resultant peptides. Betancur-Ancona *et al.* (2009), Guan *et al.* (2007) and Zhao *et al.* (2011) also found that one emulsion property increased at the expense of the other, (EAI increased, while ESI decreased). Wu *et al.* (1998) suggested the diffusion of peptides to the oil-water interface is a determining factor of EAI. The smaller the molecular size and higher the solubility, the faster the diffusion. Larger molecular size of protein, usually means slower diffusion, thus a low EAI could result. However, once larger proteins are at the interface, they slowly orient their hydrophobic moieties towards the oil, and hydrophilic towards the water, which could result in a higher stability. This could potentially explain why each enzyme has a negatively correlated EAI and ESI, as some enzymes cleave proteins into smaller peptide lengths, while some into larger peptide lengths. Vioque *et al.* (2000), found that rapeseed protein isolate hydrolyzed with Alcalase had high ESI (~68%), and lower EAI (~50%) at 3.1% DH, and low ESI (0%), and higher EAI (30%) at 7.7% DH. At the limited DH (3.1%), the stability was higher, potentially due to larger peptide length, and stability was lower at a higher DH (7.7%) potentially due to smaller peptide length. Similarly, Guan *et al.* (2007) investigated the EAI and ESI of trypsin modified oat bran concentrate and found a similar trend, were EAI increased, while the ESI decreased percent DH at all pH tested. The hydrolysates had a lower ESI compared to the control resulting from trypsin hydrolysis which increased the peptide chain length. The shorter chains of peptides are unable to stabilize the oil droplets which leads to droplet coalescence.

(d) Foaming properties

Foaming capacity (FC) and stability (FS) of untreated and hydrolyzed PPEF as a function of pH is given in Table 4.1.8. Foaming capacity refers to ability of the protein solution to incorporate air, and form a foam, whereas foam stability refers to ability of the generated foam to withstand gravity and other destabilizing factors such as coalescence. An orthogonal contrast analysis found that all hydrolyzed PPEF had lower foaming abilities than the untreated proteins, where at pH 4.0, FC was reduced from 133 to 97%, at pH 7.0 from 221 to 166%, and at pH 10.0 from 231% to 170% ($p<0.001$) (Table 4.1.2 and 4.1.8). The reduced foaming ability is thought to be associated with the lower solubility of the hydrolyzed samples, which like in emulsions would take longer to reach the air-water interface. Further analysis of the FC data showed that enzyme-type and pH, along with two, 2-way interactions (pH × DH and enzyme × pH) and a 3-way interaction (enzyme × DH × pH) were all highly significant ($p<0.001$) (Table 4.1.2). Because of the latter, the main effects cannot be discussed separately. For trypsin-treated PPEF, FC decreased with increasing levels of hydrolysis from 2-4% (FC 84%) to 10-12% (FC 31%) at pH 4.0 possibly because of its lower solubility at the higher degree of hydrolysis. At pH 7.0, solubility was similar regardless of the degree of hydrolysis leading to similar FC values (131-134%). Where at pH 10, FC improved with increased level of hydrolysis going from 155 to 202% despite having similar solubility values (Table 4.1.8). It is presumed that at the higher pH's, the presumed higher charge on the proteins would lead to faster rates of diffusion to the air-water interface so that it can form a foam upon shearing. In the case of Savinase-treated PPEF, FC followed similar trends as the trypsin within one exception. At pH 7.0, FC was slightly lower at the higher degree of hydrolysis than at the lower level which corresponded to higher levels of solubility. For papain- and pepsin-treated PPEF, FC data showed the opposite effect to trypsin and Savinase at pH 4.0. Where both enzymes led to increased FC as the degree of hydrolysis increased from 2-4% to 10-12% (Table 4.1.8). FC was relatively independent of the degree of hydrolysis at pH 7.0 and 10.0.In the case of papain, and at pH 7.0 for pepsin-treated PPEF. At pH 10.0, pepsin-treated PPEF showed increased FC as the degree of hydrolysis increased, which corresponded to a large increase in protein solubility (Table 4.1.8).

Table 4.1.8. Foaming capacity (FC%) and foaming stability (FS%) of an air-classified pea protein-enriched flour (PPEF) enzymatically modified[1] with different enzymes to achieve different level of hydrolysis at pH 4.0, 7.0 and 10.0.

Treatment	Foaming capacity (%)			Foam Stability (%)		
	pH 4.0	pH 7.0	pH 10.0	pH 4.0	pH 7.0	pH 10.0
Untreated PPEF	133 ± 6	221 ± 7	231 ± 8	9 ± 3	52 ± 3	47 ± 2
Trypsin (T) and Savinase (S)						
• Unheated (control)	21 ± 3	110 ± 9	131 ± 9	20 ± 12	72 ± 7	85 ± 8
• Heated (control)	35 ± 4	111 ± 2	158 ± 13	19 ± 6	86 ± 12	78 ± 3
• T (DH 2.3%)	84 ± 3	131 ± 5	155 ± 8	6 ± 2	85 ± 9	63 ± 3
• T (DH 10.0%)	31 ± 1	134 ± 7	202 ± 5	0 ± 0	55 ± 4	68 ± 4
• S (DH 2.3%)	78 ± 3	162 ± 7	127 ± 12	10 ± 1	70 ± 9	66 ± 3
• S (DH 10.4%)	32 ± 4	148 ± 13	189 ± 7	0 ± 0	79 ± 4	43 ± 4
Papain (Pa)						
• Unheated (control)	79 ± 7	171 ± 5	153 ± 10	9 ± 4	69 ± 4	67 ± 3
• Heated (control)	86 ± 5	187 ± 2	181 ± 12	24 ± 6	77 ± 1	72 ± 3
• Pa (DH 4.0%)	110 ± 6	178 ± 2	202 ± 10	10 ± 4	61 ± 7	70 ± 3
• Pa (DH 11.3%)	148 ± 8	191 ± 5	194 ± 14	12 ± 0	90 ± 3	76 ± 2
Pepsin (Pe)						
• Unheated (control)	112 ± 7	190 ± 17	190 ± 17	14 ± 5	78 ± 2	75 ± 9
• Heated (control)	129 ± 2	198 ± 17	206 ± 8	14 ± 3	70 ± 6	73 ± 2
• Pe (DH 2.1%)	142 ± 5	200 ± 13	212 ± 12	16 ± 2	78 ± 2	67 ± 7
• Pe (DH 10.0%)	153 ± 7	188 ± 10	172 ± 8	15 ± 2	76 ± 3	64 ± 4

[1]Enzyme treatments are as follows: a) trypsin and Savinase (pH 7.5, 37°C, 20 min, and 70 min); b) papain (pH 6.2, 45°C, 20 min and 40 min); and c) pepsin (pH 2.6, 37°C, 30 min and 70 min). All enzyme reactions were ceased by heating to 85°C for 30 min for trypsin, Savinase and pepsin, while papain was heated to 100°C for 30 min. Controls included: a) unheated (at pH values corresponding to the enzymatic treatments, but without heating) and b) heated (heated under the same temp. and pH conditions as the enzymatic treatments, but without enzymes). Upon completion all enzymatic treatments, the pea protein-enriched flour was pH adjusted to pH 7.0 prior to drying of the ingredient into a powder. All data is reported as the mean ± one standard deviation.

For FS, an orthogonal contrast analysis found that untreated and hydrolyzed PPEF at pH 4.0 had similar FS values of 9%, whereas at pH 7.0 and 10.0, FS increased from 52 to 74% and 47 to 65%, respectively with hydrolysis ($p<0.001$) (Table 4.1.2, 4.1.8). Further the analysis found that only the main effects of enzyme-type ($p<0.05$) and pH ($p<0.001$) significantly affected FS. In the case of pH, FS was found to be 9, 74 and 65% at pH 4.0, 7.0 and 10.0, respectively (Table 4.1.8). Foam stability improved at pH values away from the pI, however no further improvement was made between pH 7.0 and 10.0. In the case of enzyme-type, trypsin- and Savinase-treated PPEF behaved similarly with FS of 45-46%, whereas papain- and pepsin-treated PPEF behaved similarly with slightly better FS (53%) (Table 4.1.8).

Damodaran (2005) suggested that the larger the peptide chain length, the better the foaming capacity, and as hydrolysis decreases the peptide length, the hydrolyzed proteins decrease the foam properties. Then when hydrolyzed further, the protein could become more surface active, where it can quickly adsorb to the air-water interface, and undergo conformational changes and rearrangement to stabilize the interface. Like that of PPEF, Peragio *et al.* (1997) found that untreated pea flour had a higher foaming capacity compared to the pea flour hydrolyzed with a protease from *Aspergillus saitoi*. The decrease in the FC when hydrolyzed was attributed to the loss of soluble low-molecular weight peptides. A similar decrease in the foaming properties was observed by Yin *et al.* (2008) on trypsin-treated hemp protein isolate. An initial drop in foaming properties with partial hydrolysis (2.3% DH) was observed compared to untreated hemp protein (150% to 133.3%). Hydrolyzed samples then decreased to 122.5% at 4.5% DH, and then increased with further DH (6.7%). The FS of untreated hemp protein isolate was 58.8%, and then decreased to 44.6% after 2.3% DH and increased slightly at 4.5% DH, while further decreasing at 6.7%DH to 30.2%. The decreases in FC and FS was determined to be a result of the decrease in chain length of peptides (Damodaran, 1997).

Betancur-Ancona *et al.* (2008) found that *P. lunatus* hydrolyzed with Flavourzyme and Alcalase decreased the foaming stability compared to the untreated sample, which was like the trend observed for PPEF. The decrease in FC was thought to occur as the surface properties of the protein change, and differences to the hydrophilic/hydrophilic amino acid ratio, where a higher hydrophilic ratio was observed, favoring aqueous interactions. Similarly, the pH played an important role in the FC, where the further away the protein was from the isoelectric point, the better the FC, where the pH of both *P. lunatus* and PPEF had the lowest FC at pH 4-5. A decrease

in foaming capacity and foaming stability was observed with Protease P hydrolyzed rice bran protein in a study by Tang *et al.* (2003). The FC decreased from 47.6 to ~4.1% depending on the hydrolyzed samples drying process. The foam stability initially had a value of 78.3% and after hydrolysis no foam was observed. However, this study used a soy protein isolate as a control that is commonly used in the food industry as an ingredient and was not compared to an untreated sample of rice bran protein. Bandyopadhyay & Ghosh (2002), found a decrease in the foam stability of modified sesame protein isolates. In addition, they found that increased degree of hydrolysis did not significantly change the stability much like that of the modified PPEF in this study.

Wani *et al.* (2015) investigated the foaming properties of kidney bean protein isolate hydrolyzed with papain. The authors found an increase in foaming capacity after 30 min, and 60 min of hydrolysis. The highest foaming capacity was observed after 60 min of hydrolysis and the increases were attributed to the increase in solubility. The authors found that the lesser hydrolyzed sample had a higher percentage of foam remaining than the more hydrolyzed sample. The solubility of protein is an attributing factor in the way proteins can unfold at the water-air interface where they can reduce the surface tension. Compared to the results in this study, the solubility of PPEF decreased, therefore the ability of the hydrolyzed PPEF to unfold at the air-water interface may have been impaired. The stability of the samples in this study were lower for pepsin at all pH, trypsin and Savinase at 4.0, and 7.0, however higher for papain, and trypsin which increased at pH 10.0. The decreases in FS could be a result of the decrease in solubility, where they are unable to be adsorbed to the interface. However, the increase in FS resulting from papain-treatment may have been a result of the enzymatic mode of action, as similar increases in the stability were observed in the study by Wani *et al.* (2015).

4.1.4 Summary of physicochemical properties

In the present study, the surface and functional properties of hydrolyzed air-classified PPEF were examined with different enzymes and enzymatic conditions. The surface charge and surface hydrophobicity of the protein increased with hydrolysis as the enzymatic modification induced protein unfolding, however surface charge did not increase as much as surface hydrophobicity. The latter increased further as the degree of hydrolysis increased for all enzymes except for pepsin. It was presumed that this led to the formation of larger aggregate structures within solution, leading

to reduced solubility relative to the untreated samples, and especially at pH 4.0 which was close to the pI of the protein. As pH moved away from the pI, solubility improved due to the increased electrostatic repulsive forces between the proteins. Overall WHC, OHC and foaming stabilities were improved with hydrolysis, whereas the emulsifying capacity/stability and foaming capacity were negatively affected. However, variations in the magnitudes of these properties were seen with differences in degree of hydrolysis and pH, and with enzyme-type. The serine protease-treated PPEF (trypsin and Savinase) behaved most similarly, whereas papain- and pepsin-treated PPEF showed similar trends in the data. The similarity between trypsin- and Savinase- treated PPEF was a result of their similar reaction conditions compared to papain- and pepsin reaction conditions. Findings suggest that despite having similar degree of hydrolysis, the site of cleavage can be used as a strategy for tailoring the properties of a proteins functionality.

4.2 Nutritional properties of enzymatically modified air-classified pea protein-enriched flour treated by different enzymes to varying levels of hydrolysis

4.2.1 Bioactive compounds

Bioactive compounds have been studied for both their negative and positive impacts on the body and their influence on health. The compounds studied apart of this study are known for their ability decrease protein digestibility and bioavailability. These include total phenolic acids and condensed tannins, which can act to cross-link proteins to inhibit their digestion, and trypsin and chymotrypsin inhibitors. The concentration of these compounds found in the PPEF are given in Table 4.2.1 for untreated and hydrolyzed samples. In the case of the total phenolics an orthogonal analysis showed no difference between the untreated PPEF and hydrolyzed samples ($p>0.05$) (Table 4.2.1 and 4.2.2). Further analysis of the hydrolyzed samples revealed only the degree of hydrolysis was a significant factor affecting total phenolic concentration where the total phenolics was found to decreased from 5.9 to 5.6 mg GAE/g as the degree of hydrolysis increased from 2-4% to 10-12% ($p<0.001$). Slightly lower results were found by Han & Baik (2008), where total phenolic content was determined to be 2.5 mg GAE/g in yellow pea. Liu *et al.* (2017) investigated how *Lactobacillus* fermentation with subsequent enzymatic hydrolysis with acid protease could increase the soluble content of total phenolics from rice bran. The authors found an increase of phenolics in the supernatant of the hydrolyzed material from 1.19 to 1.89 mg GAE/g. Ti *et al.* (2015) investigated the total phenolics on cooked and enzymatically digested rice. The raw rice had a total phenolic content of ~6.5 mg/g, and when digested with pepsin and pancreatin, following an *in vitro* digestion model, the hydrolyzed protein had an increase to ~8.0 mg/g. However, cooking was found to decrease the rice to 2.0 mg/g. This could be expected in peas, however after hydrolysis occurred in the present study, the supernatant was centrifuged off, along with any solubilized total phenolic compounds that resulted from enzymatic modification would have been discarded with the supernatant. Therefore, the total phenolics measured were that of the final product. Soaking is a common method for reducing phenolics in legumes, as they can leach out in water and can be further reduced with thermal treatment when the soaking and cooking water is drained off. The decrease of phenolics with cooking is a result of the breakdown of cellular components in the peas and further release the bound phenolics (Yadav *et al.*, 2018).

Table 4.2.1. Concentration of select bioactive compounds found in an air-classified pea protein-enriched flour (PPEF) enzymatically modified with different enzymes to achieve different level of hydrolysis (DH = ~2-4% vs. ~10-12%).

Treatment[1]	Total phenolic content (mg GAE/g)	Condensed tannins (mg catechin equivalents/100g)	Trypsin inhibitors (TIU/mg)	Chymotrypsin inhibitors (CIU/mg)
Untreated PPEF	8.12 ± 0.02	0.71 ± 0.06	38.35 ± 0.78	63.86 ± 0.74
Trypsin (T) and Savinase (S)				
• Unheated (control)	7.50 ± 0.02	n.d.	13.32 ± 0.48	5.15 ± 0.14
• Heated (control)	5.18 ± 0.23	n.d.	13.47 ± 0.73	6.32 ± 0.17
• T (DH 2.3%)	5.15 ± 0.17	n.d.	11.95 ± 0.42	5.52 ± 0.12
• T (DH 10.0%)	5.70 ± 0.29	n.d.	11.00 ± 0.24	6.30 ± 0.32
• S (DH 2.3%)	5.49 ± 0.06	n.d.	10.47 ± 0.42	4.11 ± 0.15
• S (DH 10.4%)	5.79 ± 0.00	n.d.	9.87 ± 0.33	6.13 ± 0.46
Papain (Pa)				
• Unheated (control)	7.68 ± 0.04	n.d.	9.86 ± 0.54	6.23 ± 0.16
• Heated (control)	5.21 ± 0.23	n.d.	13.03 ± 0.42	6.83 ± 0.46
• Pa (DH 4.0%)	5.91 ± 0.26	n.d.	15.18 ± 0.30	3.54 ± 0.22
• Pa (DH 11.3%)	5.56 ± 0.29	n.d.	17.32 ± 0.52	6.71 ± 0.26
Pepsin (Pe)				
• Unheated (control)	7.67 ± 0.21	n.d.	22.35 ± 0.30	5.25 ± 0.15
• Heated (control)	6.97 ± 0.15	n.d.	18.52 ± 0.16	4.38 ± 0.16
• Pe (DH 2.1%)	7.08 ± 0.09	n.d.	15.48 ± 0.37	5.58 ± 0.02
• Pe (DH 10.0%)	5.41 ± 0.02	n.d.	11.00 ± 0.24	7.10 ± 0.31

[1]Enzyme treatments are as follows: a) trypsin and Savinase (pH 7.5, 37°C, 20 min, and 70 min); b) papain (pH 6.2, 45°C, 20 min and 40 min); and c) pepsin (pH 2.6, 37°C, 30 min and 70 min). All enzyme reactions were ceased by heating to 85°C for 30 min for trypsin, Savinase and pepsin, while papain was heated to 100°C for 30 min. Controls included: a) unheated (at pH values corresponding to the enzymatic treatments, but without heating) and b) heated (heated under the same temp. and pH conditions as the enzymatic treatments, but without enzymes). Upon completion all enzymatic treatments, the pea protein-enriched flour was pH adjusted to pH 7.0 prior to drying of the ingredient into a powder. All data is reported as the mean ± one standard deviation

Table 4.2.2 An individual degree of freedom (orthogonal) contrast analysis using the general linear model of the nutritional properties of untreated and enzymatically hydrolyzed pea protein-enriched flours.

Property	Untreated vs. Hydrolyzed	Enzyme-type	Degree of hydrolysis	Enzyme × Degree of hydrolysis
Bioactive compounds				
Total phenolics	NS	NS	$p<0.001$	NS
Chymotrypsin inhibitor activity	$p<0.001$	NS	$p<0.001$	NS
Trypsin inhibitor activity	$p<0.001$	$p<0.001$	NS	NS
Protein quality				
IVPD	$p<0.001$	$p<0.05$	$p<0.01$	$p<0.05$
IV-PDCAAS	NS	$p<0.01$	NS	$p<0.001$

Conditions:
Enzyme-type (trypsin, Savinase, papain, and pepsin)
pH (4.0, 7.0 and 10.0)
Degree of hydrolysis (DH) (2-4% and 10-12%)
Abbreviations:
IVPD (in vitro protein digestibility), IVPDCAAS (*in vitro* protein digestibility corrected amino acid score)

As a result of high heat or soaking, the phenolics can undergo chemical transformations, of decomposition of their structure and therefore reduced ability to complex with protein and reduce digestibility (Xu & Chang, 2008).

In the case of condensed tannins, the untreated PPEF had a level of 0.7 mg catechin equivalents/g, however it could not be detected in the hydrolyzed samples more likely due to the soaking step during the process (Table 4.2.1). These results are comparable to Wang *et al.* (1998) which found barely detectable levels (0.8 mg catechin equivalents/g) of condensed tannins in field pea. Condensed tannins in legumes vary based on the seed coat colour, where legumes with white seed coats have lower concentrations of tannins than legumes with red, or black seed coats (Troszynska & Ciska, 2002). Troszynska & Ciska (2002), found that the condensed tannin content of peas with white seed coats using the vanillin assay had undetectable levels of tannins. However, for peas with coloured seed coats, 15.6 mg/g of condensed tannins was determined using the same assay. Therefore, as peas have off-white, yellow seed coat it would be expected that peas would have low contents of condensed tannins. The initial peas were dehulled at Parrheim foods before

air classification, which removed the tannins, therefore reducing the tannins in the pea protein-enriched flour.

Even though the functional properties of legume protein hydrolysates have been studied extensively, few studies focus on the effect of enzymatic hydrolysis on the removal protease inhibitors, tannins and total phenolics. As some phenolic compounds are water soluble, during the initial soaking process the total phenolics can be extracted and removed during centrifugation (Martinez-Villaluenga *et al.*, 2009). Garcia-Mora *et al.* (2015) determined that using Savinase and Alcalase could better release total phenolic compounds from pinto beans than soaking alone. The enzymes used found that Alcalase doubled the total phenolic content after 120 min of hydrolysis, and Savinase-treated pinto beans were 2.5-times higher after 120 min of hydrolysis. In the present study, the phenolics would have been centrifuged off, and therefore, the total phenolics would have been removed with the supernatant and therefore not measured.

The control treatments (unheated and heated) reduced the trypsin and chymotrypsin inhibitors. Soaking was not performed, however overnight stirring to facilitate protein solubilization could have acted as a manner of soaking. When pulses are soaked, the trypsin and chymotrypsin inhibitors can leach into the solvent (Wang *et al.*, 1997). During the process of this study, the soaking liquid leaching the protease inhibitors was then drained off and removed, therefore removing the solubilized protease inhibitors. The concentration of trypsin inhibitor was lower in the hydrolyzed treatments (12.8 trypsin inhibitory units, TIU/mg) relative to the untreated samples (38.3 TIU/mg) ($p<0.001$). An orthogonal contrast of the hydrolyzed samples found that only enzyme-type significantly impacted trypsin inhibitory activity, where Savinase-, trypsin-, pepsin-, and papain- treated PPEF had values in increasing amounts of 10.2, 11.5, 13.2 and 16.2 TIU/mg, respectively ($p<0.001$) (Table 4.2.1, 4.2.2). Untreated PPEF had a comparable value to Wang *et al.* (1998) that determined that raw whole grass peas had a TIU/mg content range of 23.78 to 30.79 based on the cultivar and environmental conditions the peas were grown in. A TIA is considered high at ~50 TIU/mg (soybeans), therefore, peas are slightly lower than soy and with hydrolysis, decreased further, enhancing their protein quality. The concentration of chymotrypsin inhibitor was lower in the hydrolyzed treatments (5.6 chymotrypsin inhibitory units, CIU/mg) relative to the untreated samples (63.9 CIU/mg) ($p<0.001$). Gurumoorthi *et al.* (2003) determined that whole Mucuna beans had a CIA of 26.2 to 30.1 CIU/mg protein, which was considerably lower than the results found in peas in this study (63.86 CIU/mg). CIA can differ based on the

environment conditions such as drought, or cultivar. An orthogonal contrast of the hydrolyzed samples found that only the degree of hydrolysis significantly impacted chymotrypsin inhibitory activity, where inhibitory activity was increased from 4.7 to 6.6 CIU/mg as the degree of hydrolysis increased from 2-4% to 10-12%, respectively ($p<0.001$) (Table 4.2.1 and 4.2.2). Based on the findings, it was hypothesized that the enzymes could cleave some of the inhibitory proteins to lower their contents in the treated PPEF. Wang *et al.* (2008) determined that soaking cowpeas could remove 28% of the trypsin inhibitor activity, and that cooking cowpeas was even more effective at removing trypsin inhibitor activity. The authors suggested that since trypsin inhibitors are small proteins, they could be solubilized and then removed with the drained liquid. The additional decrease in TIA resulting from cooking, were attributed to the heat labile nature of trypsin inhibitor. Shi *et al.* (2017) found that soaking and cooking various Canadian pulses could decrease the chymotrypsin inhibitors significantly. Deshpande & Nielsen (1987) determined the effect of trypsin digestion and high heat on heat stable protease inhibitors in dry bean (*Phaseolus vulgaris* L.) salt- and water soluble protein fractions. Autoclaving the bean protein fractions for 5 min at 121°C could reduce both trypsin and chymotrypsin inhibitors, however they also noted that autoclaving leads to lower digestibility due to the formation of high molecular weight aggregates resulting from denaturation. The authors found that a higher enzyme substrate ratio reduced trypsin inhibitors in the fractions better than lower ratio of enzyme to substrate. Clemente *et al.* (1999) found a significant reduction in TIA (80% of the initial activity) in chickpea protein hydrolysates modified with both Alcalase and Flavourzyme to a degree of hydrolysis of >50%. Peragio *et al.* (1997) also found a significant decrease in TIA (4.72 to ~2.09 TIA/mg) when pea flour was hydrolyzed with a protease from *Aspergillus saitoi*. The reduction in TIA was suggested to result from heating during the enzyme treatment or because of the enzymatic treatment which resulted in denaturation of protein. The effect of hydrolysis on chymotrypsin inhibitors are not well known. Trypsin and chymotrypsin are similar enzyme inhibitors: both are Bowman-Birk type inhibitors, where they are composed of 71 amino acids, crosslinked by 7 disulfide bonds. The inhibitor's difference is the independent site of inhibition, where the trypsin site is Lys16-Ser17 and chymotrypsin site is Leu43-Ser44 (Birk, 1985). Trypsin targets lysine and arginine residues while chymotrypsin targets hydrophobic residues such as tyrosine, or tryptophan. The reduction in chymotrypsin inhibitors could be a result of the increased exposure of hydrophobicity as discussed in the previous section, where enzymatic hydrolysis significantly increased the surface

hydrophobicity of PPEF like that shown of Clemente *et al.* (1999). As they are both similar, low molecular weight proteins, it could be suggested that further reductions in chymotrypsin inhibitors could be reduced through enzymatic hydrolysis. Compared to whole pulse seeds, PPEF has a higher protein content which is almost double that of whole seeds. The bioactive compounds present in the pulses are associated with the protein, which could suggest why bioactive compounds present in PPEF were almost double the content of whole seeds.

4.2.2 Protein quality

Protein quality for the untreated and enzymatically modified air-classified PPEF was examined by measuring the changes to the amino acid profiles and *in vitro* digestibility. The amino acid concentration on an *as is* basis (g/100 g flour) is given in Table 4.2.3, the essential amino acid concentration (mg/g protein) in Table 4.2.3, and the amino acid score in Table 4.2.5. For all samples, the pea protein was found to be limiting in the thiol-containing amino acids: methionine and cysteine, which was expected in the case of pulse crops (Table 4.2.6) (Alizadeh & Teixeira da Silva, 2013). The limiting amino acid score is measured based on the ratio of limiting amino acid in 1 g of test protein compared to the amount in 1 g of reference protein.

Methionine composition (g per 100 g of flour, on an *as is* basis) improved with hydrolysis for pepsin-, papain-, trypsin- and Savinase-treatment at both extents of hydrolysis (2-4 and 10-12%) the cysteine content of pepsin-, papain-, trypsin- and Savinase-treatment at both extents of hydrolysis decreased. The limiting amino acid score for the untreated PPEF was found to be 0.70, where upon hydrolysis the limiting amino acid scores varied between 0.66-0.84 depending on the enzyme-type or degree of hydrolysis. The lower amino acid scores in the hydrolyzed samples than the untreated may be result of the release of peptides, which were removed from the sample during the treatment. The essential amino acid concentrations in milligrams per gram protein compared to the FAO reference protein is shown in Table 4.2.4. Untreated PPEF had lower amounts of threonine, valine, methionine, cysteine, leucine, histidine, and tryptophan compared to the FAO reference protein recommendation. Methionine and cysteine in the enzyme treated samples were found to be 4-8 mg/g protein lower than that of the FAO reference protein. Hydrolysis did not improve the methionine and cysteine contents of PPEF, and the 10-12% hydrolyzed samples were like that of the untreated sample. Threonine, valine and tryptophan contents were all found to have lower concentrations than the FAO reference amount at both extents of hydrolysis treatment.

Histidine was only lower than the FAO reference protein content from papain-treated samples and trypsin-treated samples at 10-12% DH. Isoleucine, leucine, and lysine had higher FAO reference concentrations for all enzyme-treated PPEF. Phenylalanine and tyrosine were found in much higher concentrations (12-19 mg/g) compared to the FAO reference protein.

The amino acid scores (AAS) based on the limiting amino acid are shown in Table 4.2.6. The untreated samples of PPEF had and AAS of 0.79. The AAS increased for all 10-12% DH samples, regardless of the enzyme used. However, the highest AAS was achieved with pepsin at 10-12% DH. With further hydrolysis at 10-12% DH, the samples increased compared to the 2-4% DH samples. In the case of trypsin-treated PPEF, the 2-4% DH samples had lower AAS than that of the untreated sample. The increase in AAS values in the 10-12% DH samples may have resulted from the cleavage of amino acids and unravelling of protein allowing for further breakdown of the protein and increasing the AAS. Since the extent of decrease in AAS varies amongst enzyme, it would suggest that the enzyme used affected what peptides were cleaved from the protein chain.

Villanueva *et al.* (1999) showed a similar trend in sunflower protein isolates hydrolyzed with Alcalase and Flavourzyme. The composition of amino acids in the hydrolysate are like that in the untreated sunflower protein samples. Pownall *et al.* (2010) found that Thermolysin hydrolyzed pea protein isolate had increases in hydrophobic, branched chain amino acids and positively charged amino acids. The authors attributed the changes in the pea proteins amino acid profile to the specificity of Thermolysin, where the enzyme cleaves at hydrophobic amino acids, and have the potential to release cationic amino acids. Chickpea protein isolate hydrolyzed with Alcalase and Flavourzyme decreases in phenylalanine, and arginine and the lowest amino acid contents were histidine, methionine and cysteine in a study by Clemente *et al.* (1999). The resulting changes to amino acid composition are a result of enzymatic specificity, and potential conformational characteristics that can decrease the enzymatic activity.

An orthogonal contrast found that *in vitro* digestibility (IVPD) was significantly higher for the hydrolyzed PPEF (86.4%) than the untreated samples (83.9%) ($p<0.001$) (Table 4.2.2 and 4.2.6). An orthogonal contrast of the hydrolyzed PPEF showed enzyme-type ($p<0.05$), the degree of hydrolysis ($p<0.01$) and their interaction ($p<0.05$). Since the interaction term was significant, the individual effects will not be discussed separately. IVPD for the trypsin-, papain- and pepsin-treated PPEF were independent of the degree of hydrolysis with values of 85.7%, 87.5% and 86.5%, respectively.

Table 4.2.3a Amino acid composition (g per 100 g of flour, on an *as is* basis) for untreated and, Savinase- and trypsin-treated air-classified pea protein-enriched flour (PPEF).

Amino acids	Trypsin and Savinase						
	Untreated PPEF	**Unheated pH 7.5**	**Heated pH 7.5**	**Trypsin (2.3%) DH**	**Trypsin (10.0% DH)**	**Savinase (2.3% DH)**	**Savinase (10.4% DH)**
Aspartic Acid	4.73	5.00	5.08	5.00	5.53	5.19	5.69
Glutamic Acid	7.01	7.60	7.60	7.23	7.73	7.75	8.25
Serine	2.13	2.26	2.20	2.28	2.53	2.31	2.57
Glycine	1.52	1.55	1.55	1.48	1.65	1.57	1.71
Histidine‡	0.91	1.03	1.03	0.99	0.98	1.05	1.08
Arginine	3.62	3.65	3.66	3.48	3.55	3.72	3.93
Threonine‡	1.55	1.64	1.61	1.59	1.75	1.67	1.83
Alanine	1.55	1.66	1.64	1.48	1.65	1.57	1.71
Proline	1.76	1.80	1.84	1.82	2.03	1.89	2.10
Tyrosine	1.52	1.63	1.72	1.57	1.69	1.74	1.90
Valine‡	1.49	1.96	2.08	1.83	1.85	2.00	2.07
Methionine*‡	0.44	0.50	0.52	0.49	0.60	0.50	0.60
Cysteine*	0.54	0.47	0.46	0.41	0.52	0.45	0.52
Isoleucine‡	1.45	1.94	2.03	1.80	1.88	1.98	2.06
Leucine‡	2.87	3.52	3.64	3.61	3.74	3.67	3.89
Phenylalanine‡	2.04	2.37	2.44	2.41	2.58	2.48	2.68
Lysine‡	2.95	3.32	3.29	3.19	3.45	3.36	3.69
Tryptophan‡	0.43	0.46	0.51	0.44	0.47	0.46	0.49

Notes:
*, sulfur amino acid. ‡, essential amino acids.
Measurements were performed once on each sample

Table 4.2.3b Amino acid composition (g per 100 g of flour, on an *as is* basis) for untreated and papain-treated air-classified pea protein-enriched flour (PPEF).

Amino acids	Papain				
	Untreated PPEF	**Unheated (pH 6.2)**	**Heated (pH 6.2)**	**Papain (4.0% DH)**	**Papain (11.3% DH)**
Aspartic Acid	4.73	5.13	5.21	5.08	5.54
Glutamic Acid	7.01	7.76	7.67	7.37	7.77
Serine	2.13	2.35	2.36	2.35	2.54
Glycine	1.52	1.57	1.57	1.47	1.65
Histidine‡	0.91	1.02	1.03	0.97	1.01
Arginine	3.62	3.63	3.71	3.52	3.70
Threonine‡	1.55	1.65	1.69	1.59	1.75
Alanine	1.55	1.71	1.71	1.59	1.75
Proline	1.76	1.85	1.91	1.84	2.06
Tyrosine	1.52	1.59	1.77	1.64	1.81
Valine‡	1.49	1.78	1.93	1.71	1.93
Methionine*‡	0.44	0.49	0.52	0.49	0.57
Cysteine*	0.54	0.42	0.47	0.48	0.54
Isoleucine‡	1.45	1.78	1.92	1.70	1.92
Leucine‡	2.87	3.49	3.66	3.55	3.72
Phenylalanine‡	2.04	2.35	2.49	2.38	2.61
Lysine‡	2.95	3.32	3.34	3.08	3.45
Tryptophan	0.43	0.47	0.50	0.62	0.43

Notes:
*, sulfur amino acid. ‡, essential amino acids.
Measurements were performed once on each sample

Table 4.2.3c Amino acid composition (g per 100 g of flour, on an *as is* basis) for untreated and pepsin-treated air-classified pea protein-enriched flour (PPEF).

Amino acids	**Pepsin**				
	Untreated PPEF	**Unheated (pH 2.6)**	**Heated (pH 2.6)**	**Pepsin (2.1% DH)**	**Pepsin (10.0% DH)**
Aspartic Acid	4.73	5.05	5.46	5.31	5.98
Glutamic Acid	7.01	7.33	7.75	7.37	7.08
Serine	2.13	2.31	2.42	2.34	2.26
Glycine	1.52	1.52	1.62	1.56	1.60
Histidine‡	0.91	0.98	1.06	1.04	0.99
Arginine	3.62	3.44	3.63	3.60	3.49
Threonine‡	1.55	1.62	1.75	1.74	1.71
Alanine	1.55	1.71	1.71	1.59	1.75
Proline	1.76	1.81	1.96	1.92	1.87
Tyrosine	1.52	1.67	1.83	1.89	1.69
Valine‡	1.49	1.66	1.92	1.92	1.92
Methionine*‡	0.44	0.49	0.52	0.52	0.55
Cysteine*	0.54	0.44	0.47	0.48	0.53
Isoleucine‡	1.45	1.66	1.87	1.92	2.04
Leucine‡	2.87	3.49	3.66	3.67	3.48
Phenylalanine‡	2.04	2.35	2.49	2.48	2.52
Lysine‡	2.95	3.25	3.47	3.43	3.35
Tryptophan	0.43	0.48	0.49	0.48	0.47

Notes:

*, sulfur amino acid. ‡, essential amino acids.

Measurements were performed once on each sample

Table 4.2.4 Essential amino acid concentration (mg/g) for untreated and hydrolyzed air-classified pea protein-enriched flour (PPEF).

Sample	Amino Acids								
	THR	**VAL**	**M+C**	**ILE**	**LEU**	**P+T**	**HIS**	**LYS**	**TRP**
Untreated PPEF	31	30	20	29	58	72	19	60	9
Trypsin (T) and Savinase (S)									
• Unheated pH 7.5	30	36	18	36	65	73	19	61	8
• Heated pH 7.5	30	38	18	37	67	77	19	61	9
• Trypsin 2.3%	31	35	17	35	69	76	19	61	8
• Trypsin 10.0%	32	34	21	34	69	78	18	63	9
• Savinase 2.3%	31	38	18	37	69	79	20	63	9
• Savinase 10.4%	32	37	20	36	69	81	19	65	9
Papain (Pa)									
• Unheated pH 6.2	30	33	16	32	64	72	19	61	9
• Heated pH 6.2	31	36	18	36	68	79	19	62	9
• Papain 4.0%	29	32	18	32	66	75	18	58	9
• Papain 11.3%	33	35	20	35	68	81	18	63	9
Pepsin (Pe)									
• Unheated pH 2.6	30	30	17	31	64	74	18	60	9
• Heated pH 2.6	32	35	18	34	67	79	19	63	9
• Pepsin 2.1%	33	36	19	36	69	82	19	64	9
• Pepsin 10.0%	33	40	21	40	68	82	19	66	9
FAO reference protein	34	35	25	28	66	63	19	58	11

Notes:

Abbreviations: THR (threonine); CYS (cysteine); VAL (valine); MET (methionine); ILE (isoleucine); LEU (leucine); TYR (tyrosine); PHE (phenylalanine); HIS (histidine); LYS (lysine); and TRP (tryptophan).

Measurements were performed once on each sample

Table 4.2.5 Amino acid scores for untreated and hydrolyzed air-classified pea protein-enriched flour (PPEF).

Sample	Amino Acids								
	THR	**VAL**	**M+C**	**ILE**	**LEU**	**P+T**	**HIS**	**LYS**	**TRP**
Untreated PPEF	0.93	0.86	0.79	1.05	0.88	1.15	0.98	1.03	0.79
Trypsin (T) and Savinase (S)									
• Unheated pH 7.5	0.88	1.03	0.70	1.27	0.98	1.16	1.00	1.05	0.77
• Heated pH 7.5	0.87	1.10	0.72	1.34	1.02	1.22	1.00	1.05	0.85
• Trypsin 2.3%	0.90	1.00	0.69	1.24	1.05	1.21	1.00	1.05	0.77
• Trypsin 10.0%	0.94	0.97	0.82	1.23	1.04	1.24	0.95	1.09	0.79
• Savinase 2.3%	0.92	1.07	0.72	1.33	1.05	1.26	1.03	1.09	0.79
• Savinase 10.4%	0.95	1.05	0.79	1.30	1.04	1.29	1.01	1.13	0.80
Papain (Pa)									
• Unheated pH 6.2	0.89	0.93	0.66	1.16	0.97	1.14	0.98	1.05	0.79
• Heated pH 6.2	0.92	1.02	0.73	1.28	1.03	1.26	1.01	1.07	0.85
• Papain 4.0%	0.86	0.92	0.72	1.13	1.01	1.19	0.96	1.00	0.82
• Papain 11.3%	0.98	1.01	0.81	1.26	1.03	1.29	0.97	1.09	0.85
Pepsin (Pe)									
• Unheated pH 2.6	0.87	0.87	0.68	1.09	0.97	1.17	0.95	1.03	0.80
• Heated pH 2.6	0.94	1.00	0.72	1.22	1.01	1.25	1.02	1.09	0.81
• Pepsin 2.1%	0.96	1.02	0.75	1.28	1.04	1.30	1.02	1.10	0.81
• Pepsin 10.0%	0.98	1.13	0.84	1.43	1.03	1.30	1.02	1.13	0.83

Notes:

Amino acid scores are shown as a ratio of mg of amino acid in 1 g PPEF to mg of amino acid in 1 g reference protein.

Abbreviations: THR (threonine); CYS (cysteine); VAL (valine); MET (methionine); ILE (isoleucine); LEU (leucine); TYR (tyrosine); PHE (phenylalanine); HIS (histidine); LYS (lysine); and TRP (tryptophan).

Measurements were performed once on each sample.

Table 4.2.6. Limiting amino acid scores and protein quality of untreated and hydrolyzed air-classified pea protein-enriched flours (PPEF).

Treatment	Limiting amino acid	Limiting amino acid score	*In vitro* protein digestibility (IVPD) (%)	*In vitro* protein digestibility corrected amino acid score (IVPDCAAS) (%)
Untreated PPEF	MET + CYS	0.79	83.88 ± 0.46	66.68± 0.36
Trypsin (T) and Savinase (S)				
• Unheated (control)	MET + CYS	0.70	86.17 ± 0.42	60.71 ± 0.29
• Heated (control)	MET + CYS	0.72	86.17 ± 0.54	62.56 ± 0.39
• T (DH 2.34)	MET + CYS	0.69	85.47 ± 0.39	59.17 ± 0.27
• T (DH 10.02%)	MET + CYS	0.82	85.99 ± 0.05	70.53 ± 0.04
• S (DH 2.31%)	MET + CYS	0.72	85.56 ± 0.09	62.13 ± 0.06
• S (DH 10.39%)	MET + CYS	0.79	86.50 ± 0.22	68.60 ± 0.18
Papain (Pa)				
• Unheated (control)	MET + CYS	0.66	85.39 ± 0.16	56.31 ± 0.10
• Heated (control)	MET + CYS	0.73	87.53 ± 0.43	64.16 ± 0.31
• Pa (DH 3.96%)	MET + CYS	0.72	86.26 ± 0.20	62.45 ± 0.15
• Pa (DH 11.34%)	MET + CYS	0.81	88.76 ± 0.05	72.11 ± 0.04
Pepsin (Pe)				
• Unheated (control)	MET + CYS	0.68	86.48 ± 0.18	59.19 ± 0.12
• Heated (control)	MET + CYS	0.72	86.51 ± 0.10	62.24 ± 0.08
• Pe (DH 2.12%)	MET + CYS	0.75	86.48 ± 0.31	64.57 ± 0.23
• Pe (DH 10.03%)	MET + CYS	0.84	86.51 ± 0.21	72.89 ± 0.18

[1]Enzyme treatments are as follows: a) trypsin and Savinase (pH 7.5, 37°C, 20 min, and 70 min); b) papain (pH 6.2, 45°C, 20 min and 40 min); and c) pepsin (pH 2.6, 37°C, 30 min and 70 min). All enzyme reactions were ceased by heating to 85°C for 30 min for trypsin, Savinase and pepsin, while papain was heated to 100°C for 30 min. Controls included: a) unheated (at pH values corresponding to the enzymatic treatments, but without heating) and b) heated (heated under the same temp. and pH conditions as the enzymatic treatments, but without enzymes). Upon completion all enzymatic treatments, the pea protein-enriched flour was pH adjusted to pH 7.0 prior to drying of the ingredient into a powder. Amino acid scores are shown as a ratio of mg of amino acid in 1 g PPEF to mg of amino acid in 1 g reference protein. IVPD and IVPDCAAS data is reported as the mean ± one standard deviation.

In contrast, Savinase-treated PPEF had IVPD values that increased from 85.6 to 86.5% as the degree of hydrolysis increased from 2-4% to 10-12%, respectively (Table 4.2.6). Enzyme pre-treatment of lentil flour, concentrates and isolates with acid protease, papain prior to digestion studies was shown to increase the digestibility in a study performed by Aryee & Boye (2016). The increases were observed when protein concentration increased in the sample where flour had the lowest digestibility and isolates had the highest, suggesting that the processing conditions and removal of a certain portion of carbohydrates could have increased the access of the digestive enzymes to the protein (Betancur-Ancona *et al.,* 2009). The authors also suggested that acid protease (pepsin) and papain enzyme treatment provided mild lentil protein structure modification which could have enhanced the digestive enzymes access to peptide bonds (Aryee & Boye, 2016) which also occurred in the present study. With an extensively hydrolyzed chickpea protein (>50%), Clemente *et al.* (1999) found a very high IVPD (>96%) for Alcalase, Flavourzyme, and Alcalase-Flavourzyme combination. The authors suggested that the extensive hydrolysis favours the digestion of protein, resulting from the liberation of shorter chain peptides. In a study by Dias *et al.* (2010), they determined that enzyme-treated bean flour with *Bacillus* sp. protease (Savinase), and trypsin increased the digestibility of the flour. The highest increase in digestibility are a result from Savinase treatment. The increases in digestibility are attributed to the increase in liberation of amino acids and short chain peptides, which can be more readily digested. The increases in IVPD are observed for all hydrolysates compared to the control, regardless of the enzyme. This could have been a result from the removal of bioactive compounds which may inhibit the digestion process. Tavano *et al.* (2008) suggested that the increase in *in vitro* protein digestibility for the heated samples compared to the untreated may be less reflective of the inactivation of heat labile bioactive compounds but more because of conformational changes. Phenolics, trypsin and chymotrypsin inhibitors are reduced with soaking and further with hydrolysis, and as they interfere with protein digestion, their reduction enhanced their digestibility.

An orthogonal contrast on *in vitro* protein digestibility corrected amino acid scores (IV-PDCAAS) between untreated and hydrolyzed PPEF was found not to be significant ($p>0.05$) (Table 4.2.2 and 4.2.6), however analysis of the hydrolyzed samples showed enzyme-type ($p<0.01$) and its interaction with degree of hydrolysis ($p<0.001$) to be significant. For each enzyme, IV-PDCAAS increased as the degree of hydrolysis increased from 2-4% and 10-12% although the magnitude of those increases differed. For trypsin-, papain- and pepsin-treated PPEF, IV-

PDCAAS had similar increases in magnitude, going from 59.1% to 70.5%, 62.4% to 71.1%, and 64.5% to 72.9%, respectively. In the case of Savinase-treated PPEF, IV-PDCAAS had a smaller increase from 62.1% to 68.6% (Table 4.2.6). Hydrolysis breaks the peptide bond between two amino acids, creating two smaller protein chain sequences. Hydrolyzed protein based diets are prescribed for patients with gastrointestinal issues where digestion is impaired as it is suggested that hydrolysates have better intestinal absorption compared to untreated proteins and therefore have better digestibility (Zieglar *et al.*, 1998).

Tavano *et al.* (2016) compared the differences between *in vitro* and *in vivo* protein digestibility techniques on chickpea flours, and protein fractions for calculating PDCAAS. Comparing *in vitro* protein digestibility to the *in vivo* method, the chickpea flour and protein fractions have a lower digestibility percent when using the *in vitro* method except for the glutelin fraction. After incorporation of the amino acid score, the *in vitro* PDCAAS still have a lower value than the *in vivo* method. The *in vitro* method in most cases over-estimates the digestibility of protein as it does not fully resemble the characteristics of digestion: the initial stomach conditions (pH, and pepsin), and the gastric movements. Even if no digestion of protein occurred (no pH drop), the digestibility will be 65.66%, which is an overestimation. Additionally, if the pH drops below 6.1, the IVPD can be above 100% (Tinus *et al.*, 2102). Tavano *et al.* (2008) noted that during *in vivo* studies, the extent of digestibility was not affected by the presence of anti-nutritional factors, but rather changes in the pancreas of the rats were observed which could potentially have been an adaptation to the high concentration of trypsin inhibitors present.

4.2.3 Summary of nutritional properties

Many researchers have found that soaking decreased the levels of phenolics, trypsin inhibitors and chymotrypsin inhibitors with whole seeds (Mulimani & Supriya, 1994; Vidal-Valverde *et al.,* 1994; Khandelwal *et al.,* 2010). Although soaking was not performed per say, the enriched flour was dispersed in water and heated during the enzymatic treatments, prior to collecting the modified PPEF afterwards. During this process, some of these compounds is thought to be lost. In the present study, levels of bioactive compounds were reduced substantially. The slight rise in digestibility of the proteins may be the result of the release of small peptide chain is (di, or tri-peptides), which has previously been shown to improve the ability of the corn gluten meal protein to be digested and absorbed (Jin *et al.,* 2014). The improvements may also be the

result of a presumed reduction in protein size and/or opening of the protein conformation as the result of hydrolysis, and the reduction in bioactive compounds that are known to inhibit digestion. All treatments were found to be limiting in methionine and cysteine, as expected for pulses, however variability was introduced with the amino acid scores upon hydrolysis as small peptides were presumed cut off and removed during the process. As a result, IV-PDCAAS became overall worse for the hydrolyzed PPEF samples than the untreated ones.

5. OVERALL CONCLUSIONS

Estimations by the FAO and the UN suggest that the global population will reach 9 billion people by 2050. As the population increases, the world's production of food must also increase by 70% to meet supply demands. A strategy for meeting growing demand is increasing the production of leguminous crops as they are a nutrient dense crop, and are a common crop grown around the world. Legumes are low cost and have favorable agronomic practices (*i.e.*, fixes soil nitrogen and less water usage). Peas (*Pisum sativum*) are a leguminous crop grown in western Canada, where Saskatchewan had the largest growing area (Bekkering, 2011). A significant portion of legumes grown in Canada are exported to countries such as China, India and Turkey (Bekkering, 2011). Legumes grown in Canada are a significant ingredient in the canning industry and incorporated into food products when used as food ingredients (*i.e.*, flours, enriched flours, dry/wet concentrates, isolates and hydrolysates). Pea flour is sometimes isolated and modified to add value by reducing bioactive compounds that have adverse health effects and increasing the nutritional protein, and aid in improving the functionality of the ingredient. The present research evaluated the impact of enzymatic hydrolysis on an air-classified pea protein-enriched flour using four proteolytic enzymes, at two extents of hydrolysis. The hydrolyzed samples of PPEF were evaluated based on the physicochemical, functional and nutritional properties, to determine the compatibility in food aid products and use as an ingredient in the food industry.

To prepare the hydrolyzed PPEF, the initial starting material was hydrolyzed to two different extents of hydrolysis; 2-4% DH and 10-12% DH, using trypsin, pepsin, papain and Savinase. A part of this research focused on the physicochemical composition, and functional properties. Hydrolysis altered the proximate composition of the PPEF, where protein concentration increased, while the lipid and ash contents decreased after hydrolysis. The alteration in the distribution of composition were thought the be attributed to centrifugation after hydrolysis, where enzyme treatment could release bound lipids from the protein chain and remain in the supernatant which is then drained off, along with minerals. The surface and functional properties of the hydrolyzed PPEF were examined, and found that hydrolysis did not increase the zeta potential as

it did with surface hydrophobicity resulting from the unfolding of protein. Surface hydrophobicity increased with an increasing extent of hydrolysis, apart from pepsin hydrolyzed samples, where the surface hydrophobicity remained at~46 A.U. The solubility of the hydrolyzed PPEF samples decreased compared to the starting material. The decreases in solubility was presumed to be a result of larger aggregate structure forming, leading to reduced solubility. Solubility of the hydrolyzed PPEF samples was especially low at pH 4.0, which can be attributed to the pH being near the pI of the protein, and as the pH increased away from the pI, electrostatic and repulsive forces between the proteins could act and better solubilize the protein. The WHC and OHC and foaming stabilities improved with hydrolysis. However, emulsifying capacity and stability and foaming capacity decreased with hydrolysis. Even though these properties decreased with hydrolysis, there were significant differences observed in the degree of hydrolysis, pH and enzyme choice. Trypsin- and Savinase- (serine proteases) treated PPEF had comparable properties, while papain and pepsin-treated PPEF had similar trends to each other. The current findings show that even though the hydrolyzed samples had the same range of degree of hydrolysis, the specificity of the enzymes and its preferred site of cleavage can be used to modify proteins functionality.

Traditional preparation of pulses involves soaking the seeds in water, and current research has found that soaking can aid in decreasing the levels of phenolics, trypsin inhibitors and chymotrypsin inhibitors (Mulimani & Supriya, 1994; Vidal-Valverde *et al.*, 1994; Khandelwal *et al.*, 2010). Soaking on whole seeds was not performed, however PPEF was dispersed in water and left to stir overnight as a form of solubilization, and then heated during the enzymatic treatments, before centrifugation to collect protein and freeze dried. During this process, the bioactive compounds were thought to be lost in the supernatant. In the current research, the bioactive compounds were reduced significantly. The decreases in bioactive compounds increased the digestibility of the proteins as a result of less enzyme inhibitors, and interfering compounds also. Digestibility could have increased because of the liberation of small peptides (di, or tri-peptides), which have been suggested by Jin *et al.* (2014) to increase hydrolyzed corn gluten meal protein ability to be digested and absorbed. The reduction in protein size, and conformational changes to the protein structure from enzymatic hydrolysis may be responsible for the improvements in digestibility, as well as a reduction in bioactive compounds, known to inhibit digestion. Methionine and cysteine in limiting amounts were expected in pulses, and all treatments were unable to change the limiting amino acid score. The inability to change the limiting amino acid

score lead to a decrease in the IV-PDCAAS for hydrolyzed PPEF samples compared to untreated PPEF, with the exception of the 10-12% DH papain-, pepsin- and trypsin-treated samples, which had IV-PDCAAS of 72.1%, 72.9%, and 72.5%, respectively. These samples attained the IV-PDCAAS value required (70%) which can be used as a food aid ingredient for moderately malnourished children as specified by the WHO.

Enzymatic treatment using trypsin, pepsin, papain and Savinase improved certain functional properties, while impairing others. Depending on the intended use of the final product, enzymatic treatment can be employed as a feasible method for protein modification. In the case of solubility, enzymatic hydrolysis decreased the proteins solubility in water at pH 4, 7, and 10 at 2-4%, however an improvement at 10-12% was observed for pepsin. All enzymatic treatments improved the OHC and WHC with the most observable improvements occurring with papain at 10-12% DH. Enzymatic treatments on PPEF decreased the EAI and ESI, and FC and FS which was attributed to the decrease in solubility, and low zeta potential. For bioactive compounds; enzymatic hydrolysis could decrease phenolics, tannins, and protease inhibitors, however, minimal increases in digestibility were observed. Based on the intended use of the ingredient, the enzyme choice can aid in improving the product's oil and water holding capacity and increasing the protein quality of PPEF.

6. FUTURE STUDIES

This research investigated the effects of enzymatic modification on the physicochemical, functional and nutritional properties of PPEF. The enzymes chosen for hydrolysis were all food grade. However, many authors reported using a combination of enzymes for hydrolysis with varying specificities, showing promising results in modifying protein with good solubility and increased functional properties (Clemente *et al.*, 1999; Cumby *et al.*, 2007; Thamnarathip *et al.,* 2016). Exploring different combinations of the four enzymes used in this study, and a variety of conditions (*i.e.*, temperature, pH and enzyme substrate ratio) could be examined further in order to create a high quality PPEF with a variety of increased functional properties. For example, as solubility increased with a higher degree of hydrolysis and as many functional properties depended on the solubility, increasing the degree of hydrolysis to 20-50% could enhance the solubility and therefore the resultant functional properties. This could be achieved by altering the enzyme substrate ratio, or in combination with another enzyme.

The functional properties that were studied would be of use for determining the type of food formulation that hydrolyzed PPEF could be applied to. The oil and water holding capacity improved with hydrolysis, that suggests that adding hydrolyzed PPEF into processed meat systems, or in other baked goods may be beneficial. Formulation trials with hydrolyzed PPEF as well as sensory analysis would further aid in determining its applications and at what concentrations it can be incorporated into products.

Since this research focused on air-classified pea protein-enriched flour, a more concentrated protein such as an isolate, and even into a globulin-rich isolate, could provide greater control over the hydrolysis process. However, when concentrating or purifying the protein, more waste could be formed resulting from the removal of starch and other carbohydrates. Since carbohydrates are a significant portion of the overall composition (~40%), determining the starch and fibre content could determine how they affect the functional properties such as gelation and pasting. Additionally, determining the fibre content could provide a more thorough look at the digestibility of PPEF as a high concentration of fibre can result in impaired nutrient adsorption.

There are many other antinutritional properties associated with pulses that could impair protein digestion such as hemagglutinins, saponins, phytate, and lectins. Determining their contents in pulses could provide a more thorough understanding their acceptability in food-aid related products.

There are multiple methods of measuring protein digestibility, and it is important to determine if both *in vitro* protein digestibility methods and *in vivo* protein digestibility methods are comparable. *In vivo* animal studies involve a rat bioassay to determine PER, true fecal digestibility, PDCAAS and DIAAS, and as they involve the use of a living animal with a comparable digestive system, they could obtain a closer result to human systems. However there are ethical concerns regarding the use of animals in experiments, and high costs leads to a challenge in obtaining a reliable way of measuring digestibility *in vivo*. Therefore, having a dependable and comparable way of measuring the digestibility using *in vitro methods* is needed.

Building on from the current study, the presence of certain peptides have shown that there might be properties that could enhance health for example; such as ACE-inhibitory peptides (Roy *et al.,* 2010). Enzymatic hydrolysis is a highly-controlled process where it cleaves at specific locations, and can potentially release certain peptide sequences if the enzyme is tailored to a specific sequence. Therefore, this method can be used to produce inhibitory peptides that can lead to health promoting effects; such as ACE-inhibitory peptides. ACE-inhibitory peptides have been studied to reduce hypertension and have been found in pepsin, and pancreatin hydrolyzed plant materials (Megias *et al.,* 2004). Accordingly, PPEF peptides could be alternatively marketed as nutraceutical products.

REFERENCES

AACC International (2000). *Approved methods of the AACC International:* Method 22-40.01 (10th ed.) St. Paul, MN, USA: American Association of Cereal Chemists.

Achouri, A., Boye, J. I., Yaylayan, V. A., & Yeboah, F. K. (2005). Functional properties of glycated soy 11*S* Glycinin. *Food Chemistry and Toxicology, 70*(4)*,* 269-274.

Alizadeh, K., & Teixeira da Silva, J. A. (2013). Mixed cropping of annual feed legumes with barley improves feed quantity and crude protein content under dry-land conditions. *Maejo International Journal of Science and Technology*, *7*(1), 42-47.

AOAC. (2005). Official methods of analysis (16th ed.) Washington D.C. Association of Official Analytical Chemists.

Adler-Nissen, J. (1979). Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. *Journal of Agricultural and Food Chemistry, 27*(6)*,* 1256-1262.

Adler-Nissen, J. (1984). Control of the proteolytic reaction and of the level of bitterness in protein hydrolysis processes. *Journal of Chemical Technology and Biotechnology, 34*(3)*,* 215-222.

Aryee, A. N. A., & Boye, J. I. (2016). Improving the digestibility of lentil flours and protein isolate and characterization of their enzymatically prepared hydrolysates. *International Journal of Food Properties, 19*(12)*,* 2649-2665.

Avramenko, N. A., Low, N., & Nickerson, M. T. (2013). The effects of limited enzymatic hydrolysis on the physicochemical and emulsifying properties of a lentil protein isolate. *Food Research International, 51*(1)*,* 162-169.

Aviles-Gaxiola, S., Chuck-Hernandez, C., & Serna Saldivar, S. O. (2017). Inactivation methods of trypsin inhibitor in legumes: a review. *Journal of Food Science, 83*(1), 17-29

Banerjee, S., & Bhattacharya, S. (2012). Food Gels: Gelling Process and New Applications. *Food Science and Nutrition, 52*(4)*,* 334-346.

Barac, M., Cabrilo, S., Pesic, M., Stanojevic, S., Pavlicevic, M., Macej, O., & Ristic, N. (2011). Functional properties of pea (*Pisum sativum, L.*) protein isolates modified with chymosin. *International Journal of Molecular Science, 12*(12), 8372-8387.

Barac, M., Pesic, M., Stanojevis, S., Kostic, A., & Cabrilo, S. (2015). Techno-functional properties of pea (*Pisum sativum)* protein isolates:a review. *Acta Periodica Technologica, 46*(46), 1-18.

Bekkering, E. (2014). Pulses in Canada. *Canadian Agriculture at a glance, Statistics Canada.* Retrieved from https://www150.statcan.gc.ca/n1/en/catalogue/96-325-X201400114041.

Betancur-Ancona, D., Martinez-Rosado, R., Corona-Crus, A., Castellanos-Ruelas, A., Jaramillo-Flores, M., & Chel-Guerrero, L. (2017). Functional properties of hydrolysates from *Phaseolus lunatus* seeds. *International Journal of Food Science and Technology, 44*(1), 128-137.

Bandyopadhyay, K., & Ghosh, S. (2002). Preparation and characterization of papain-modified sesame (*Sesamum indicum* L.) protein isolates. *Journal of Agricultural and Food Chemistry, 50*(23), 6854-6857.

Bidlingmeyer, B. A., Cohen, S. A., Tarvin, T. L., & Frost, B. (1987). A new, rapid, high-intensity analysis of amino acids in food type samples. *Journal of AOAC International, 70*(2), 241-247.

Birk, Y. (1985). The Bowman-Birk inhibitor. Trypsin- and chymotrypsin-inhibitor from soybeans. *International Journal of Peptide and Protein Research, 25*(2), 113-131.

Blytt, H. J., Guscar, T. K., & Butler, L. G. (1988). Antinutritional effects and ecological significance of dietary condensed tannins may not be due to binding and inhibiting digestive enzymes. *Journal of Chemical Ecology, 14*(6), 1455-1465.

Boye, J. I., Aksay, S., Roufik, S., Ribereau, S., Mondor, M., Farnworth, E. R., & Rajamohamed, S. H. (2010). Comparison of the functional properties of pea, chickpea, and lentil concentrates processed using ultrafiltration and isoelectric precipitation. *Food Research International, 43*(2), 537-546.

Boye, J., Zare, F., & Pletch, A. (2010). Pulse proteins: Processing, characterization, funcitonal properties and applications in food and feed. *Food Research International, 43*(2), 414-431.

Bradford, M., M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*(1-2)*,* 248-254.

Brune, M., Rossander, L, & Halberg, L. (1989). Iron absorption and phenolic compounds: importance of different phenolic structures. *European Journal of Clinical Nutrition, 43*(8)*,* 547-557.

Cai, L., Xiao, L., Liu, C., & Ying, T (2013). Functional properties and bioactivities of pine nut (*Pinus gerardiana*) protein isolates and its enzymatic hydrolysates. *Food and Bioprocess Technology, 6*(8)*,* 2109-2117.

Can Karaca, A., Nickerson, M. T., & Low, N. H. (2011). Lentil and chickpea protein stabilized emulsions: optimization of emulsion formulation. *Journal of Agricultural and Food Chemistry, 59*(24)*,* 13203-13211.

Chang, C., Tu, S., Ghosh, S., & Nickerson, M. T. (2015). Effect of pH on the inter-relationships between the physicochemical, interfacial, and emulsifying properties for pea, soy, lentil and canola protein isolates. *Food Research International, 77*(3)*,* 360-367.

Chavan, J. K., Kadam, S. S., Salunkhe, D. K., & Beuchat, L., R. (1986). Biochemistry and technology of chickpea (*Cicer arietinum* L.) *Critical Reviews in Food Science and Nutrition, 25*(2)*,* 107-158.

Chen, B., McClements, D. J., Gray, D. B., & Decker, E. (2010). Stabilization of soybean oil bodies by enzyme (laccase) cross-linking of adsorbed beet pectin coatings. *Journal of Agricultural and Food Chemistry, 58*(16), 9259-9265.

Clemente, A. (2000). Enzymatic protein hydrolysates in human nutrition. *Trends in Food Sceince & Technology, 11*(7), 254-262.

Clemente, A., Vioque, J., Sanchez-Vioque, R., Pedroche, J., & Millan, F. (1999). Production of extensive chickpea (*Cicer arietinum* L.) protein hydrolysates with reduced antigenic activity. *Journal of Agricultural and Food Chemistry, 47*(9)*,* 3776-3781.

Clemente A., Vioque, J., Sanchez-Vioque, R., Pedroche, J., Bautista, J. & Millan F. (1999) Protein quality of chickpea (*Cicer arietinum* L.) protein hydrolysates. *Food Chemistry, 67*(3)*,* 269-274.

Cui, C., Zhao, M., Yuan, B., Zhang, Y., & Ren, J. (2013). Effect of pH and pepsin limited hydrolysis on the strucutre and functional properties of soybean protein hydrolysates. *Journal of Food Science, 78*(12)*,* 1871-1877.

Cumby, N., Zhong, Y., Naczk, M., & Shahidi, F. (2008). Antioxidant activity and water holding capacity of canola protein hydrolysates. *Food Chemistry, 109*(1)*,* 144-148.

Damodaran, S. (2005) Protein stabilization of emulsions and foams. *Journal of Food Science*, *70*(3)*,* R54-R66.

D'Archivio, M., Filesi, C., Di Benedetto, R., Gargiulo, R., Giovannini, C., & Masella, R. (2007). Polyphenols, dietary sources and bioavailability. *Annali dell'Istituto Superiore di Sanita, 43*(4)*,* 348-361.

Day, L. (2013). Proteins from land plants- potential resources for human nutrition and food security. *Trends in Food Science and Technology, 32*(1)*,* 25-42.

Del Mar Yust, M., Pedroche, J., del Carmen Millan-Linares, M., Alcaide-Hidalgo, J. M., & Francisco, M. (2010). Improvement of functional properties of chickpea proteins by hydrolysis with immobilized Alcalase. *Food Chemistry, 122*(4)*,* 1212-1217.

Deng, L., Wang, Z., Yang, S., Song, J., Que, F., Zhang, H., & Feng, F. (2016). Improvement of functional properties of wheat gluten using acid protease from *Aspergillus usamii. PLoS One, 11*(7)*,* 1-13.

Deshpande, S. & Nielsen. (1987). *In vitro* digestibility of dry bean (*Phaseolus vulgaris* L.) proteins: the role of heat-stable protease inhibitors. *Journal of Food Science, 52*(5)*,* 1330-1334.

Dias, D. R., Maria Patto de Abreu, C., Pinto Coelho Silvestre, M., & Freitas Schwan, R. (2010). *In vitro* protein digestibility of enzymatically pre-treated bean (*Phaseolus vulgaris* L.) flour using commercial protease and *Bacillus* sp. protease. *Food Science and Technology, 30*(1)*,* 94-99.

Diaz, A. M., Caldas, G. V., & Blair, M. W. (2010). Concentration of condensed tannins and anthocyanins in common bean seed coats. *Food Research International, 43*(2)*,* 595-601.

Faller, A. L. K., & Fialho, E. (2009) The antioxidant capacity and polyphenol content of organic and conventional retail vegetables after domestic cooking. *Food Research International, 42*(1)*,* 210-215.

FAO. (2013). The state of food insecurity in the world: the multiple dimensions of food security. Retrieved from: http://www.fao.org/docrep/018/i3434e/i3434e.pdf.

FAO/WHO. (2011). *Dietary protein quality evaluationn in human nutrition.* Auckland: FAO/WHO.

FAO. (1991). The state of food and agriculture. Retrieved from http://www.fao.org/3/a-t0496e.pdf

Foegeding, E. A., & Davis, J. P. (2011). Food Protein Functionality: A comprehensive approach. *Food Hydrocolloids, 25*(8), 1853-1964.

Fredrikson, M., Biot, P., Alminger, M. L., Carlson, N. G., & Sandberg, A. S. (2001). Production process for high quality pea-protein isolates with low content of oligosaccharides and phytate. *Journal of Agricultural and Food Chemistry, 49*(3), 1208-1212.

Fuhrmeister, H., & Meuser, F. (2003). Impact of processing on functional properties of protein products from wrinkled peas. *Trends in Food Science and Technology, 56*(2-3), 119-129.

Garcia-Mora, P., Frias, J., Penas, E., Zielinski, H., Gimenez-Bastida, J., Wiczkowski, W., Zielinska, D., & Martinez-Villaluenga, C. (2015) Simultaneous release of peptides and phenolics with antioxidant, ACE-inhibitory and anti-inflammatory activities from pinto bean (*Phaseolus vulgaris* L. var. pinto) proteins by subtilisins. *Journal of Functional Foods, 18*(A), 319-332.

Ghribi, A. M., Gafsi, I. M., Sila, A., Blecker, C., Danthine, S., Attia, H., Bougatef, A., & Besbes, S. (2015). Effects of enzymatic hydrolysis on conformational and functional properties of chickpea protein isolate. *Food Chemistry, 187,* 322-330.

Govindaraju, K., & Srinivas, H. (2006). Studies on the effects of enzymatic hydrolysis on funcitonal and physicochemical properties of arachin. *LWT- Food Science and Technology, 39*(1), 54-62.

Grimble, G. (1994). The Significance of Peptides in Clinical Nutrition. *Annual Review Nutrition, 14,* 419-447.

Gueguen, J. (1983). Legume seed protein extraction, processing, and the end product characteristics. *Plant Foods for Human Nutrition, 32*(3-4), 267-303.

Guan, X., Yao, H., Chen, Z., Shan, L., & Zhang, M. (2008) Some functional properties of oat bran protein concentrate modified by trypsin. *Food Chemistry, 101*(1), 163-170.

Gurumoorthi, P., Pugalenthi, M., & Janardhanan, K. (2003). Nutritional potential of five accessions of a south Indian tribal pulse *Mucuna pruriens* var utilis: II. Investigations on

total free phenolics, tannins, trypsin and chymotrypsin inhibitors, phytohaemagglutinins, and *in vitro* protein digestibility. *Tropical and Subtropical Agroecosystems, 1,* 153-158.

Hall, G.M. *Methods of testing protein functionality;* Blackie Academic & Professional: London, UK, 1996; p 265.

Han, H., & Baik, B. (2008). Antioxidant activity and phenolic content of lentils (*Lens culinaris),* chickpeas (*Cicer arietinum* L.) peas (*Pisum sativum* L.) and soybeans (*Glycine max*) and their quantitative changes during processing. *International Journal of Food Science & Technology, 43*(11)*,* 1971-1978.

Humiski, L. M., & Aluko, R. E. (2007). Physiochemical and bitterness properties of enzymatic pea protein hydrosylates. *Journal of Food Science, 72*(8)*,* 605-611.

Isaschar-Ovdat, S., Rosenberg, M., Lesmes, U., & Fishman, A. (2015). Characterization of oil-in-water emulsions stabilized by tyrosinase-crosslinked soy Glycinin. *Food Hydrocolloids, 43,* 493-500

Jiang, J., Xiong, Y. L., & Chen, J. (2010). pH shifting alters solubility characterisitics and thermal stability of soy protein isoalte and its globulin fractions in diferent pH, salt concentration and temperature conditions. *Journal of Agricultural and Food Chemistry, 58*(13)*,* 8035-8042.

Jin, J., Ma., Zhou, C., Luo, M., Liu, W., Qu, W., He, R., Luo, L., & Yagoub, A. (2014). Effect of degree of hydrolysis on the bioavailability of corn gluten meal hydrolysates. *Journal of the Science of Food and Agriculture, 19*(12)*,* 2501-2509.

Jood, S., Chauhan, B., & Kapoor, C. (1986). Saponin content of chickpea and black gram: Varietal differences and effects of processing and cooking methods. *Journal of the Science of Food and Agriculture, 37*(11)*,* 1121-1124.

Jung, S., Murphy, P., & Johnson, L. (2005) Physicochemical and functional properties of soy protein substrates modified by low levels of protease hydrolysis. *Journal of Food Science, 70*(2)*,* 180-187.

Karamac, M., Amarowicz, R., & Kostura, H. (2002). Effect of temperature and enzyme/substrate ratio on the hydrolysis of pea protein isolate by trypsin. *Czech Journal of Food Science, 20*(1)*,* 1-6.

Kasera, R., Singh, A., Prasad, K., & Arora, N. (2015). Enzymatic hydrolysis: A method in alleviating legume allergenicity. *Food and Chemical Toxicology, 76,* 54-60.

Kato, A. & Nakai, S. (1980). Hydrophobicity determined by fluorescence probe methods and its correlation with surface properties of proteins. *Biochimica et Biophysica Acta, 624*(1)*,*13-20.

Khalid, I. I., & Elhardallou, S. B. (2015). The effect of pH on foaming properties of cowpea (*Viguna ungiculata* L. walp) flour and protein isolates. *Nutrition and Food Sciences, 5*(4)*,* 1-7

Khandelwal, S., Udipi, S., & Ghurge, P. (2010). Polyphenols and tannins in Indian pulses: Effect of soaking, germination and pressure cooking. *Food Research International, 43*(2)*,* 526-530.

Kimura, A., Fukuda, M., Zhang, M., Motoyama, S., Maruyama, N., & Utsumi, S. (2008). Comparison of physiochemical properties of 7*S* and 11*S* globulins from pea, fava bean, cowpea, and french bean with those of soybean - French bean 7*S* exhibits excellent properties. *Journal of Agricultural and Food Chemistry, 56*(21)*,* 10273-10779.

Kiosseoglou, V., & Paraskevopoulou, A., Functional and physiochemical properties of pulse protein. In: Tiwari BK, Gowen, A., Mckenna, B., editors. Pulse Foods Processing: Quality & Nutritional application. San Diego: Academic Press: 2011. 57-89.

Klupsaite, D., & Juodeikiene, G. (2015). Legume: composition, protein extraction and functional properties. A review. *Chemical Technology, 66*(1)*,* 5-12.

Kong, X., Zhou, H, & Qian, H. (2007). Enzymatic preparation and functional properties of wheat gluten hydrolysates. *Food Chemistry, 101*(2)*,* 615-620.

Kramer, R. M., Shende, V. R., Motl, N., Pace, C. N., & Scholtz, J. M. (2012). Toward a molecular understanding of protein solubility: increased negative surface charge correlates with increased solubility. *Biophysical Journal, 102*(8)*,* 1907-1915.

Ladjal-Ettoumi, Y., Boudries, H., Chibane, M., & Romero, A. (2016). Pea, Chickpea and Lentil protein isolates: Physicochemical characterization and emulsifying properties. *Food Biophysics, 11*(1)*,* 43-51.

Laemmli, U. K. (1970). Glycine-SDS-PAGE for separation of proteins. *Nature, 227*, 680-685.

Lam, A. C. Y., Warkentin, T. D., Tyler, R. T., & Nickerson, M. T. (2017) Physicochemical and functional properties of protein isolates obtained from several pea cultivars. *Cereal Chemistry, 94*(1)*,* 89-97.

Lamsal, B. P., Jung, S., & Johnson, L. A. (2007). Rheological properties of soy protein hydrolysates obtained from limited enzymatic hydrolysis. *LWT-Food Science and Technology, 40*(7)*,* 1215-1223.

Le Gall, M., Gueguen, J., Seve, B., & Qullien, L. (2005). Effects of grinding and thermal treatment on hydrolysis susceptibility of pea proteins (*Pisum sativum* L.). *Journal of Agricultural and Food Chemistry, 53*(8)*,* 3057-3064.

Liu, L., Zhang, R., Deng, Y., Zhang, Y., Xiao, J., Huang, F., Wen, W. & Zhang, M. (2017). Fermentation and complex enzyme hydrolysis enhance total phenolics and antioxidant activity of aqueous solution from rice bran pre-treated by steaming with α-amylase. *Food Chemistry, 221,* 636-643.

Lopez-Barrios, L., Gutierrez-Uribe, J. A., & Serna-Saldivar, S. O. (2014). Bioactive peptides and hydrolysates from pulses and their potential use as functional ingredients. *Journal of Food Science, 79*(3)*,* R273-R283.

Makkar, H., Siddhuraju, P., & Becker, K. (2007). *Methods in molecular biology: Plant secondary metabolites* (pp. 1-27). Totowa: Humana Press Inc.

Martinez-Villaluenga, C., Zielinski, H., Frias, J, Piskula, M. K., Kozlowsha, C. & Vidal-Valverde, C. (2009). Antioxidant capacity and polyphenolic content of high-protein lupin products. *Food Chemistry, 112*(1)*,* 84-88.

Maurer, R. W., Sandler, S. I., & Lenhoff, A. M. (2011). Salting-in characteristics of globular proteins. *Biophysical Chemistry, 156*(1)*,* 72-78.

McClements, D. (2004). Protein-stabilized emulsions. *Current Opinion in Colloid & Interface Science, 9*(5)*,* 305-313.

McClements, D. (2007). Critical review of techniques and methodologies for characterization of emulsion stability. *Critical Reviews in Food Science and Nutrition, 47*(7)*,* 611-649.

Megias, C., Pedroche, J., Yust, M. M., Giron-Calle, J., Alaiz, M., Millan, F., & Vioque, J. (2008) Production of copper-chelating peptides after hydrolysis of sunflower proteins with pepsin and pancreatin. *Journal of Agricultural and Food Chemistry, 55*(16), 6509-6514.

Meinlschmidt, P., Sussmann, D., Schweiggert-Weisz, U., & Eisner, P. (2016). Enzymatic treatment of soy protein isolates: effects on the potential allergenicity, technofunctionality and sensory properties. *Food Science and Nutrition, 4*(1)*,* 11-23.

Mession, J. L., Chihi, M. L., Sok, N., & Saurel, R. (2015). Effect of globular pea proteins fractionation on their heat-induced aggregation and acid cold-set gelation. *Food Hydrocolloids, 46,* 233-243.

Miedzianka, J., Peksa, A., Rytel, E., Tajner-Czopek, A., & Kita, A. (2014) Improving the properties of fodder potato protein concentrate by enzymatic hydrolysis. *Food Chemistry, 159,* 512-519.

Mulimani, V. H., & Supriya, D. (1994) Tannic acid content in sorghum (*Sorghum biocolour* M.): Effects of processing. *Plant Foods for Human Nutrition, 46*(3) 195-200.

Mune Mune, M. (2015). Optimizing functional properties of bambara bean protein concentrate by enzymatic hydrolysis using pancreatin. *Journal of Food Processing and Preservation, 39*(6)*,* 2572-2580.

Nesse, K. O., Nagalakshmi, A. P., Marimuthu, P., & Singh, M. (2011). Efficacy of a fish protein hydrolysate in malnourished children. *Indian Journal of Clinical Biochemsitry, 26*(4)*,* 360-365.

O'Kane, F. E., Happe, R. P., Vereijken, J. M., Gruppen, H., & Van Boekel, M. A. (2004). Heat-induced gelation of pea legumin: comparison with soybean glycinin. *Journal of Agricultural and Food Chemistry, 52*(16)*,* 5071-5078.

Owusu-Ansah, Y., & McCurdy, S. (1991). Pea proteins: A review of chemistry, technology of production, and utilization. *Food Review International, 7*(1)*,* 103-134.

Ozdal, T., Capanoglu, E. & Altay, F. (2013). A review on protein-phenolic interactions and associated changes. *Food Research International, 51*(2)*,* 954-970.

Panyam, D., & Kilara, A. (1996). Enhancing the functionality of food proteins by enzymatic modification. *Trends in Food Science & Technology, 7*(4)*,* 120-125.

Pearce, K. N. & Kinsella, J. E. (1978). Emulsifying properties of proteins: Evaluation of a turbidimetric technique. *Journal of Agricultural and Food Chemistry, 26*(3)*,* 716-723.

Pelgrom, P. J. M., Vissers, A. M., Boom, R. M., & Schutyser, M. A. I. (2013). Dry fractionation for the production of functional pea protein concentrates. *Food Research International, 53*(1)*,* 232-239.

Perez-gago, M. B., & Krochta, J. M. (2001). Denaturation time and temperature effects on solubility, tensile properties and oxygen permeability of whey protein edible films. *Journal of Food Science, 66*(5)*,* 705-710.

Periago, J. M., Vidal, L. M., Ros, G., Rincon, F., Martinez, C., & Lopez, G. (1998). Influence of enzymatic treatment on the nutritional and functional properties of pea flour. *Food Chemistry, 63*(1), 71-78.

Polanco-Lugo, E., Davila-Ortiz, G., Betancur-Acona, D., & Chel-Guerro, L. A. (2014). Effects of sequential enzymatic hydrolysis on structural, bioactive and functional properties of Phaseolus lunatus protein isolate. *Food Science and Technology, 34*(3), 441-448.

Pownall, T. L., Udenigwe, C. C., & Aluko, R. E. (2010). Amino acid composition and antioxidant properties of pea seed (*Pisum sativum* L.) enzymatic protein hydrolysate fractions. *Journal of Agricultural and Food Chemistry, 58*(8), 4712-4718.

Price, M. L., Van Scoyoc, S., & Butler, L. G. (1978). A critical evaluation of the vanillin reaction as an assay for tannin in sorghum grain. *Journal of Agriculture and Food Chemistry, 26*(5), 1214-1218.

Pulse Canada. (2016). *Food & Health*. Retrieved from Pulse Canada: http://www.pulsecanada.com/food-health.

Rackis, J., Wolf, W., & Baker, E. (1986). Protease inhibitors in plant foods: content and inactivation. M. Friedman (Ed.), Advances in Experimental Medicine and Biology: Nutritional and Toxicological Significance of Enzyme Inhibitors in Foods, Plenum Press, New York. pp. 299-337.

Reichert, R. D. (1982). Air Classification of peas (*Pisum sativum*) varying widely in protein content. *Journal of Food Science, 47*(4), 1263-1267

Renekema, J. M. S., Lakemond, C. M. M., de Jongh, H. H. J., Gruppen, H., & van Vilet, T. (2000). The effect of pH on heat denaturation and gel forming properties of soy properties. *Journal of Biotechnology, 79*(3), 223-230.

Rubio, L. A., Perez, A., Ruiz, R., Guzman, M. A., Aranda-Olmedo, I., & Clemente, A. (2014). Characterization of pea (*Pisum sativum*) seed protein fractions. *Journal of the Science of Food and Agriculture, 94*(2), 280-287.

Sabate, J., & Soret, S. (2014). Sustainability of plant based diets: back to the future. *American Journal of Clinical Nutrition, 100*(1), 476S-482S.

Sarwar, G. (1997). The Protein Digestibility–Corrected Amino Acid Score method overestimates quality of proteins containing antinutritional factors and of poorly digestible proteins supplemented with limiting amino acids in rats. *The Journal of Nutrition, 127*(5), 758-764.

Scalbert, A., Manach, C., Morand, C., Remesy, C., & Jimenez, L. (2005). Dietary polyphenols and the prevention of diseases. *Critical Review of Food Science and Nutrition, 45*(4)*,* 287-306.

Schnepf, M. (2013). Protein-Water Interactions. In B. Hudson, *Biochemistry of Food Proteins* (pp. 1-12). 2013: Elsevier Applied Science.

Sherwy, P. R., Napier, J. A., & Tatham, A. S. (1995). Seed storage proteins: structures and biosynthesis. *American Society of Plant Physiologists, 7*(7)*,* 945-956.

Shi, L., Mu, K., Arntfield, S. D., & Nickerson, M. (2017). Changes in levels of enzyme inhibitors during soaking and cooking for pulses available in Canada. *Journal of Food Science and Technology, 54*(4)*,* 1014-1022.

Singhal, A., Can Karaca, A., Tyler, R. & Nickerson, M. (2016) Pulse Proteins: From Processing to structure-function relationships. A.K. Goyal (Ed.), Grain Legumes, Intech (2016).

Singleton, V., & Rossi, J. (1965) Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology Viticulture, 16,* 144-158.

Soares, S., Vitorino, R., Osorio, R., Fernandes, A., Venancio, A., & Mateues, N. (2011). Reactivity of human salivary proteins families toward food polyphenolics. *Journal of Agricultural and Food Chemistry, 59*(10)*,* 5535-5547.

Sosulski, F. & Youngs, C. G. (1979). Yield and functional properties of air-classified protein and starch fractions from eight legume flours. *Journal of the American Oil Chemists' Society, 56*(3)*,* 292-295.

Stone, A. K., Karalash, A., Tyler, R. T., Warkentin, T. D., & Nickerson M. T. (2015). Functional attributes of pea protein isolated prepared using different extraction methods and cultivars. *Food Research International, 76*(1)*,* 31-38.

Sun, X. D. (2011). Enzymatic hydrolysis of soy proteins and the hydrolysates utilization. *Food Science and Technology, 46*(12)*,* 2447-2459.

Sun, X. D., & Arntfield, S. D. (2010). Gelation properties of salt-extracted pea protein induced by heat treatment. *Food Research International, 43*(2), 509-515.

Tamm, F., Herbst, S., Brodkorb, A., & Drusch, S. (2016). Functional properties of pea protein hydrolysates in emulsions and spray dried microcapsules. *Food Hydrocolloids, 58*(C)*,* 204-214.

Tang, S., Hettiarachchy, N. S., Horax, R., & Eswaranandam, S. (2003). Physicochemical properties and functionality of rice bran protein hydrolysate prepared from heat-stabilized

defatted rice bran with the aid of enzymes. *Food Chemistry and Toxicology, 68*(1)*,* 152-157.

Tavano, O. L. (2013). Protein hydrolysis using proteases: An important tool for food biotechnology. *Journal of Molecular Catalysis B: Enzymatic, 90,* 1-11.

Tavano, O. L., Neves, V. A., & da Silva Junior, S. I. (2012) *In vitro* versus *in vivo* protein digestibility techniques for calculating PDCAAS (protein digestibility-corrected amino acid score) applied to chickpea fractions. *Food Research International, 89*(1)*,* 756-763.

Thamnarathip, P., Jangchud, K., Jangchud, A., & Vardhanabhuti, B. (2016). Functional properties of protein hydrolysates from Riceberry rice bran. *International Journal of Food Science and Technology, 51*(5)*,* 1110-1119.

Ti, H., Zhang, R., Li, Q., Wei, Z., & Zhang, M. (2015). Effects of cooking an *in vitro* digestion of rice on phenolic profiles and antioxidant activity. *Food Research International, 76*(3)*,* 813-820.

Tinus, T., Damour, M., Van Riel, V., & Sopade, P. A. (2012). Particle size-starch-protein digestibility relationships in cowpea (*Vigna unguiculata*). *Journal of Food Engineering,113*(2), 254-264.

Tirgar, M., Silcock, P., Carne, A., & Birch, J.E. (2017). Effect of extraction method on functional properties of flaxseed protein concentrates. *Food Chemistry, 215,* 417-424.

Troszynska, A. & Ciska, E. (2002). Phenolic compounds of seed coats of white and coloured varieties of pea (*Pisum sativum* L.) and their total antioxidant activity. *Czech Journal of Food Sciences, 20*(1)*,* 15-22.

Tsoukala, A., Papalamprou, E., Makri, E., Doxastakis, G., & Braudo, E. E. 2006) Adsorption at the air-water interface and emulsification properties of grain legume protein derivatives from pea and broad bean. *Colloids and Surfaces B:Biointerfaces, 53*(2)*,* 203-208.

Tzitzakas, E. N., Vincken, J. P., De Groot, J., Gruppen, H., & Visser, R. G. (2006). Genetic variation in pea seed globulin composition. *Journal of Agricultural and Food Chemistry, 54*(2)*,* 425-433.

Utsumi, S., Matsumura, Y., & Mori, T. (1997). Structure-function relationships of soy proteins. In *Food Proteins and Their Interactions,* Damodaran, D, Paraf, A., Eds.;Marcel Dekker: New York 257-291.

Vankosky, M. A., Carcamo, H. A., McKenzie, R. H., & Dosdall, L. M.(2011). Integrated management of *Sitona lineatus* with nitrogen fertilizer, Rhizobium and thiamethoxan insecticide. *Agronomy Journal, 103*(3)*,* 565-572.

Ventureira, J., Martinez, E. N., & Anon, M. C. (2010). Stability of oil: Water emulsions of amaranth proteins. Effect of hydrolysis and pH. *Food Hydrocolloids, 24*(6-7)*,* 551-559.

Vidal-Valverde, C., Frias, J., Estrella, I., Gorospe, M. J., Ruiz, R., & Bacon, J. (1994). Effect of processing on some antinutritional factors of lentils. *Journal of Agriculture and Food Chemistry, 42*(10), 2291-2295.

Villanueva, A., Vioque, J., Sanchez-Vioque, R., Clemente, A., Pedroche, J, Bautista, J., & Millan, F. (1999*). Journal of the American Oil Chemists Society, 76*(12), 1455-1460.

Vioque, J., Sanchez-Vioque, R., Clemente, A., Pedroche, J., & Millan, F. (2000) Partially hydrolyzed rapeseed protein isolates with improved functional properties. *Journal of the American Oil Chemists Society, 77*(4)*,* 447-450.

Wani, I. A., Sogi, D. S., Shivare, U. S., & Gill, B. S. (2015). Physico-chemical and functional properties of native and hydrolyzed kidney bean (*Phaseolus vuglaris* L.) protein isolates. *Food Research International, 76*(1)*,* 11-18.

Wang, N., Hatcher, D. W. & Gawalko, E. J. (2008). Effect of variety and processing on nutrients and certain anti-nutrients in field peas (*Pisum sativum*). *Food Chemistry, 111*(1)*,* 132-138.

Wang, N., Lewis, M. J., Brennan, J. G., & Westby, A. (1997). Effect of processing methods on nutrients and antinutritional factors in cowpea. *Food Chemistry, 58*(1-2), 59-68.

Wang, X., Warkentin, T. D. , Briggs, C. J., Oomah, B., Campbell, C. G.,. & Woods, S. (1998). Total phenolics and condensed tannins in field pea (*Pisum sativum* L.) and grass pea (*Lathyrus sativus* L.). *Euphytica, 101*(1)*,* 97-102.

Wilde, P. J. (2000). Interfaces: their role in foam and emulsion behaviour. *Current Opinion in Colloid & Interface Science, 5*(3-4)*,* 176-181.

Wilde & Clark (1996). Hall, G.M. (Ed.) Methods of testing protein functionality, Blackie Academic and Professional, London. pp. 110-152.

Wilde, P. J., Mackie, A., Husband, F., Gunning, P., & Morris, V. (2004). Proteins and emulsifiers at liquid interfaces. *Advances in Colloid and Interface Science, 108-109,* 63-71.

Wu, W. U., Hettiarachchy, N. S., & Qi, M. (1998). Hydrophobicity, solubility, and emulsifying properties of soy protein peptides prepared by papain modfication and ultrafiltration. *Journal of the American Oil Chemists Society, 75*(7)*,* 845-850.

Xu, B., & Chang, S. K. C. (2008). Effect of soaking, boiling, and steaming on total phenolic content and antioxidant activities of cool season food legumes. *Food Chemistry, 110*(1), 1-13

Yadav, N., Kaur, D., Malaviya, R., Singh, M., Fatima, M., & Singh L. (2018). Effect of thermal and non-thermal processing on antioxidant potential of cowpea seeds. *International Journal of Food Properties, 21*(1), 437-451

Yin, S, W. Tang, C. H., Cao, J. S., Hu, E. K., Wen, Q. B., & Yang, X. Q. (2008). Effects of limited enzymatic hydrolysis with trypsin on the functional properties of hemp (Cannabis sativa L.) protein isolate. *Food Chemistry, 106*(3)*,* 1004-1013.

Zayas, J. (1997). Foaming Properties of Proteins. In J. Zayas, *Functionality of Proteins in Foods* (pp. 260-309). New York: Springer Berlin Heidleberg.

Zayas, J. (1997). Oil and fat binding properties of proteins. In J. Zayas, *Functionality of Protein in Foods* (pp. 228-259). New York: Springer Berlin Heidelberg.

Zayas, J. (1997). Water Holding Capacity of Proteins. In J. Zayas, *Functionality of Proteins in Foods* (pp. 76-133). New York: Springer Berlin Heidelberg.

Zeng, M., Adhikari, B., He, Z., Qin, F., Huang, X., & Chen, J. (2013). Improving foaming properties of soy protein isolate through partial enzymatic hydrolysis. *Drying Technology, 31*(13-14)*,* 1545-1552.

Zhao, G., Liu, Y., Zhao, M., Ren, J., & Yang, B. (2011) Enzymatic hydrolysis and their effects on conformational and functional properties of peanut protein isolate. *Food Chemistry, 127*(4)*,* 1438-1443.

Zhour, J. R. & Erdman Jr, J. W. (1995). Phytic acid in health and disease. *Critical reviews in Food Science and Nutrition, 35*(6)*,* 495-508.

Ziegler, F., Nitenberg, G., Coudray-Lucas, C., Lasser, P., Giboudeau, J., & Cynober, L. (1998). Pharmacokinetic assessment of an oligopeptide-based enteral formula in abdominal surgery patients. *American Journal of Clinical Nutrition, 67*(1), 124-128